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CONTENTS

PART 1. MARCH, 1943

PAGE

G. ANDERSON AND A. E. NORTH: "The Relation of Pertussis Endotoxin to Pertussis Immunity in the Mouse" 1

HENRY FRANCIS HOLDEN: "The Determination of Small Amounts of Carbon Monoxide in Air and Blood" 9

NANCY ATKINSON: "Antibacterial Substances Produced by Moulds. 2. The Antibacterial Substances Produced by Some Common Penicillia" 15

A. W. TURNER AND A. W. RODWELL: "The ϵ Toxin of *Cl. Welchii* Type D. 1. Proteolytic Conversion of ϵ Prototoxin into ϵ Toxin by Trypsin and Other Proteases" 17

A. W. TURNER AND A. W. RODWELL: "The ϵ Toxin of *Cl. Welchii* Type D. 2. Mechanism of its Development in Cultures through the Action of Extracellular Proteinases upon ϵ Prototoxin" 27

J. G. WOOD, D. H. CRUICKSHANK AND R. H. KUCHEL: "The Metabolism of Starving Leaves. 1. Presentation of Data; The Nature of Respiration Rate/Time Curves in Air and in Nitrogen and the Relation to Carbohydrates. 2. Changes in Amounts of Total and Chloroplast Proteins, Chlorophyll, Ascorbic Acids and Soluble Nitrogen Compounds. 3. Changes in Malic and Citric Acid Contents and Inter-Relations of these with Soluble Nitrogen Compounds" 37

PART 2. JUNE, 1943

F. M. BURNET AND DIANA R. BULL: "Changes in Influenza Virus Associated with Adaptation to Passage in Chick Embryos" .. 55

F. M. BURNET AND W. I. B. BEVERIDGE: "Titration of Antibody Against Influenza Viruses by Allantoic Inoculation of the Developing Chick Embryo" 71

A. W. TURNER AND CATHARINE E. EALES: "Studies on the *Clostridium Oedematiens* Group. 1. 'H' and 'O' Antigenic Analysis" .. 79

B. J. GRIEVE: "Studies in the Physiology of Host-Parasite Relations. 4. Some Effects of Tomato Spotted Wilt on Growth" 89

ELLEN CLARK AND F. P. O. NAGLER: "Haem-Agglutination by Viruses. The Range of Susceptible Cells with Special Reference to Agglutination by Vaccinia Virus" 103

J. DAVIDSON AND D. C. SWAN: "The Incubation Period of the Eggs of *Halotydeus Destructor* Tucker (Acarina) at Different Temperatures" 107

J. W. H. LUGG AND R. A. WELLER: "Germinating Seeds as a Source of Vitamin C in Human Nutrition. 1. Ascorbic and Dehydroascorbic Acid Contents of Several Varieties of Seeds Germinating under Standard Conditions for Varying Periods of Time" 111

E. WESTON HURST, BARBARA TERNENT COOKE AND P. MELVIN: "'Nervous Distemper' in Dogs. A Pathological and Experimental Study, with Some Reference to Demyelinating Diseases in General" 115

NANCY ATKINSON: "Antibacterial Substances Produced by Moulds. 3. The Detection and Estimation of Antibacterial Activity *in vitro*" 127

PART 3. SEPTEMBER, 1943

ALFRED GOTTSCHALK: "d-Fructo-Pyranose, a Sugar Unfermentable by Yeast" 133

ALFRED GOTTSCHALK: "The Proportion of Fructo-Furanose present in d-Fructose Solution at Equilibrium (0° C.)" 139

PAGE

E. WESTON HURST AND BARBARA TERNENT COOKE: "Capillary Fat-Embolism in the Brains of Sheep, Pigs and Monkeys, with especial reference to Demyelination and other Lesions in the White Matter" 141

E. WESTON HURST, BARBARA TERNENT COOKE, JESSICA MAWSON AND P. MELVIN: "Nuclear Inclusions developing in the Kidneys of Australian Opossums maintained under Laboratory Conditions" 149

JOAN B. CLELAND: "The Effect of Salicylates, (A) on the Estimation of Thiamine by the Thiochrome Method, (B) on the Excretion of Thiamine" 153

HENRY FRANCIS HOLDEN: "The Blood Pigment and Cyanides; a Green Haemoglobin" 159

HENRY FRANCIS HOLDEN: "A Method for the Colorimetric Determination of Oxyhaemoglobin" 169

NANCY ATKINSON: "A New Salmonella Type: *Salmonella Adelaide*" .. 171

ROBERT CECIL BETTY AND VICTOR MARTIN TRIKOJUS: "Hypericin and a Non-Fluorescent Photosensitive Pigment from St. John's Wort (*Hypericum Perforatum*)" 175

PATRICIA KEOGH AND F. H. SHAW: "The Pharmacology and Toxicity of Alstonia Alkaloids" 183

W. K. WHITTEN: "Some Observations on the Excretion of Keto-Steroids and Oestrogens in the Urine of Sheep" 187

PART 4. DECEMBER, 1943

A. H. K. PETRIE AND J. I. ARTHUR (compiled by J. G. WOOD): "Physiological Ontogeny in the Tobacco Plant. The Effect of Varying Water Supply on the Drifts in Dry Weight and Leaf Area and on various Components of the Leaves" 191

N. S. TIVER AND R. F. WILLIAMS: "Studies of the Flax Plant. 2. The Effect of Artificial Drought on Growth and Oil Production in a Linseed Variety" 201

J. W. H. LUGG AND R. A. WELLER: "Germinating Seeds as a Source of Vitamin C in Human Nutrition. 2. The Germinating and Processing of Blue Boiler Peas (*Pisum Sativum*) as a Palatable Source of Vitamin C" 211

A. M. CLARK: "Some Physiological Functions of the Nucleus in Amoeba, investigated by Micrurgical Methods" 215

MARY H. PETHERICK AND E. SINGER: "The Influence of Metals on Diphtheria Infection" 221

F. M. BURNET: "Characteristics of the Influenza Virus-Antibody Reaction as tested by the Method of Allantoic Inoculation" 231

R. LEMBERG: "Transformation of Verdohaemochromogens into Monoaza-Haemins" 239

NANCY ATKINSON AND NEVILLE F. STANLEY: "Antibacterial Substances Produced by Moulds. 4. The Detection and Occurrence of Suppressors of Penicidin Activity" 249

NANCY ATKINSON AND NEVILLE F. STANLEY: "Antibacterial Substances Produced by Moulds. 5. The Mechanism of the Action of Some Penicidin Suppressors" 255

F. H. S. ROBERTS: "Observations on *Anopheles Annulipes* Walk. as a possible vector of Malaria. 1. The Relative Susceptibility of *An. Annulipes* and *An. Punctulatis* var. *Moluccensis* Sw. to Experimental Infection with Malaria Parasites" 259

THE RELATION OF PERTUSSIS ENDOTOXIN TO PERTUSSIS IMMUNITY IN THE MOUSE

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(From the Commonwealth Serum Laboratories, Melbourne).

(Accepted for publication 22nd January, 1943.)

Many workers have emphasized the probable significance of pertussis endotoxin in the pathology and immunology of whooping cough. Teissier *et al.* (1929) obtained pertussis antitoxin by immunizing animals with formolized toxin, and Evans (1940) confirmed and extended these findings.

Convalescent sera, effective in prophylaxis of pertussis infection of the lung in humans (Paterson *et al.*, 1935; Bradford, 1935; Meader, 1937; Cohen and Lapin, 1939) and in mice (North *et al.*, 1939) contain no antitoxin, and the effect of such sera has been attributed therefore to antibacterial properties (North *et al.*, 1939). Becker (1939) obtained unfavourable results with pertussis antitoxin used in the prophylaxis and treatment of whooping cough.

Burnet and Timmins (1937) showed that the mouse is susceptible to lung infection by *H. pertussis* following intranasal inoculation of a virulent strain, and that the pathology of the infection resembles that of whooping cough in humans. The rôle of pertussis toxin in immunity of mice to lung infection was investigated by observing the effect on the course of the lung infection in mice of (i) attempted active immunization with formolized toxin and (ii) attempted passive protection by antitoxin. In the course of the work other points of interest emerged.

MATERIALS AND METHODS.

Cultures. Phase I strains virulent for mice by intranasal inoculation were used.

Preparation of toxins. Toxins were prepared after the method of Evans (1940). Saline suspensions of 48-hour cultures on Bordet-Gengou (B.G.) medium were strained through fine gauze, centrifuged and the organisms re-suspended in water. This suspension was placed in an aluminium container and alternately frozen (in solid CO_2 and alcohol) and thawed (in water at 30°) 25–30 times for periods of 10 minutes. The suspension was dried from the frozen state and ground in an agate mortar for half an hour. On culture, the dried organisms and even the dried and ground organisms gave an abundant growth. The ground organisms were extracted with saline (5 mg. per ml.) for 1 hour and after centrifuging in an angle centrifuge for 1 hour supernates free from living *H. pertussis* were obtained.

Standardization of toxins. The lethal effect of the toxin when given intravenously to mice was used for standardization in preference to the skin necrotic effect in the rabbit, used by Evans. When a toxin was titrated on the skins of 5 rabbits, free from agglutinins for *B. bronchisepticus*, the end point of the titration and the degree of blanching, reddening and necrosis varied. When given intravenously to mice in a volume of 0·2 ml. a clean-cut end point was usually obtained with 50 p.c. increments of dosage (Table 1, toxin controls). Toxins regularly contained 1 mouse M.L.D. in 0·2 ml. when diluted 1: 15 or 1: 20. Given intraperitoneally the toxin had an M.L.D. approximately 1½ times as great as when given intravenously. The M.L.D. for 20 gm. mice was ½ of the M.L.D. for 400 gm. guinea-pigs, injected intracardially.

TABLE 1.

Standardization of antitoxins in mice.

Antitoxin.	M.L.D. of toxin in test dose.	Antitoxin in test dose (ml.) 1/10	1/20	1/40	1/80	1/160	1/320	Mouse units. per ml. antitoxin.
R3	5	s	s	s	s	+	+	400
R4	5	s	s	s	s	+	+	400
R8	5	s	s	s	+	+	+	200
R28	5	s	s	s	s	+	+	400
R19	2½	+	+	+	+	−	−	150

Toxin Controls.

Mice received 0·2 ml. I.V. of dilutions indicated	1/5	1/7	1/10	1/15	1/20
	+	+	+	s	s

s = mouse survived. + = mouse died.

Ground bacteria kept in stoppered tubes at room temperature deteriorated in toxin content in the course of weeks, as had the toxin used in Table 1; but preparations sealed in vacuo in glass and kept at 2° C. lost no toxicity in 12 months.

Production and standardization of antitoxin. Antitoxins were prepared by subcutaneous inoculation of rabbits at weekly intervals of toxin treated with 0·2 p.c. formalin for 7 days at 37° C. Five rabbits were each given three injections of 1 ml. of formolized toxin followed by two injections of 2 ml. The five antitoxins obtained by bleeding these rabbits were standardized by neutralization of both the skin necrotic effect of the toxin in the rabbit and its lethal effect when given intravenously to mice. In rabbit standardizations the technique of Evans (1940) was used. In mouse standardizations, animals were given intravenously a constant dose of toxin of about 5 M.L.D. plus graded amounts of antitoxin. The toxin-antitoxin mixtures were allowed to react at room temperature for 1½ hours, and the volume of each animal dose was adjusted to 0·2 ml. Three mice were used for each toxin-antitoxin dose. Both methods of standardization placed the five antitoxins in the same ratios of potency, the results of the mouse standardization being given in Table 1. We have preferred the mouse standardization method and, since our preparations were intended for immunity experiments in mice, it seemed best to have them standardized in terms of mouse units.

Intranasal infecting method of testing immunity in mice. Experimental technique and criteria of immunity were those developed by Burnet and Timmins (1937), North *et al.* (1939), North *et al.* (1941). Mice were infected intranasally with small (5×10^3) or large (50×10^6) infecting doses of *H. pertussis*; and serum, when used, was given intraperitoneally 5 or 6 hours before the infecting dose. Those mice which survived a specified period, usually one week, were killed and the degree of specific pertussis lung infection in each mouse was assessed according to the density of growth obtained after smearing the cut surfaces of the lungs over B.G. plates. Degree of lung infection was graded as previously (North *et al.*), viz.: 4 = confluent growth on plates; 3 = semiconfluent growth; 2 = many discrete colonies; 1 = few colonies; 0 = no growth. The statistical significances of experimental results were evaluated by the tables of Loewenthal and Wilson (1940) on the basis of either (i) the proportion of mice surviving for a specified time after infection; or (ii) the proportion of mice having lungs clear of infection after a specified time, as shown by lung culture.

IMMUNIZATION OF MICE WITH FORMOLIZED TOXIN.

Five groups of 20 mice were given respectively 1, 2, 3, 4 and 5 injections, each of 0·1 ml. of formolized toxin. The injections were given at intervals of 5 days and were so arranged that the five series of injections were completed simultaneously. Fourteen days after the last injection, 15 mice from each group, together with 15 non-immunized controls, were tested for immunity to a large intranasal infecting dose of *H. pertussis*.

TABLE 2.

Survival rates of mice immunized with (A) formolized toxin or (B) phase I vaccine, and subsequently infected intranasally.

	Doses of formolized toxin.	No. of survivors after—days.					Intensity of lung infection in survivors.*
		1 day	2	3	4	21	
A	5	15	13	9	8	5	00000
	4	15	11	7	7	7	0000000
	3	15	8	4	4	3	000
	2	15	9	6	4	2	00
	1	15	9	7	5	2	00
	Nil	15	3	1	0	0	
B	Dose of vaccine						
	10^9 organisms	20	17	16	14		
	Nil	20	3	1	1		

* Survivors were killed 21 days after infection and their lungs cultured.
All the mice received a large intranasal infecting dose (50×10^6 organisms).

The results are given in Table 2A. Comparison of the death rates of control and immunized mice four days after infection reveals a significant immunity in those mice which received 1, 2 and 3 doses of formolized toxin and a highly significant immunity in those which received 4 and 5 doses. The degree of immunity produced in the mice by 4 and 5 doses of formolized toxin was of the same order as that produced by immunization with Phase I vaccine. For comparison the survival rate of mice immunized with one dose of vaccine, containing 10^9 organisms, and subsequently given the same intranasal infecting dose is shown in Table 2B. In view of the high antitoxin response obtained in rabbits (see Table 1) to the identical batches of formolized toxin

that were used on the mice, the results in Tables 2A and 2B did not suggest that any new element of immunity had appeared in the mice immunized with that material; but rather that the immunity obtained with it was due to a bacterial antigen present in the toxin.

The five remaining mice from each of the groups immunized with formolized toxin were tested for immunity to intravenous doses of toxin, ranging from 1 to 5 M.L.D.: all the mice died. To confirm this finding that formolized toxin provoked little or no antitoxic response in the mouse, two groups of 20 mice were given five injections of formolized toxin, each of 0·1 ml., one group being dosed intraperitoneally, and the other subcutaneously. Eight days after the last dose the mice were injected intravenously, with doses of toxin ranging from ½ to 2 M.L.D. No significant difference was found in the M.L.D. of the toxin for the immunized mice and for non-immunized controls. In addition, 5 mice from each of the immunized groups were bled out. The two lots of pooled sera so obtained failed to protect mice when amounts up to 0·2 ml. were combined with 1½ M.L.D. of toxin and injected intravenously. We have therefore been unable to detect any antitoxic response in mice to repeated doses of relatively large amounts of formolized toxin.

PASSIVE PROTECTION BY ANTITOXIN AGAINST INTRANASAL INFECTION WITH *H. PERTUSSIS*.

(a) *Unabsorbed antitoxin.* Using twelve groups of 6 mice, a preliminary comparison was made of the protective action of the 5 rabbit antitoxins and of normal rabbit serum, in doses of 0·3 ml., against both large and small intranasal infecting doses of *H. pertussis*. Although doses of antitoxin up to 120 mouse units were given, none exerted a protective effect significantly better than that of normal rabbit serum. There was clearly no new marked protection due to antitoxin.

A comparison was next made, with groups of ten mice, of the protective property of antitoxin R28 and of a standard convalescent serum of proven protective value (North *et al.* 1939). The results (Table 3) show that both sera gave a significant protection of the same order. Since the convalescent serum contained no antitoxin detectable either by titration in the mouse or in the skin of a rabbit, the protection conferred by the sera could not be related to their antitoxin content.

TABLE 3.

Protective action of antitoxin and of convalescent serum against intranasal infection.

Serum given.	Antitoxin units.	Mouse deaths.	Mouse lungs infected	Intensity of lung infection in survivors.
R28 (0·3 ml.)	120	0/10	3/10	4, 3, 2
AC8 (0·3 ml.)	Nil	0/10	3/10	3, 2, 1
Nil	Nil	1/10	9/10	4, 4, 2, 2, 2, 2, 1, 1, 1

All the mice received a small intranasal infecting dose (5,000 organisms). Survivors were killed 7 days later and cultures made of their lungs.

The possibility that antitoxin might be more efficacious if given in divided doses over a period of time was investigated. Two groups of ten mice were injected with 120 units of antitoxin per mouse and given a small infecting dose intranasally. One group of mice received the antitoxin in one dose six hours before infection, whilst the other group received it in three doses of 40 units, given respectively twenty-four hours before, three hours before, and three days after the infecting organisms. The results showed no superiority in favour of the antitoxin given in divided doses.

(b) *Absorbed antitoxin.* Evans (1940) found that he could remove agglutinins from his sera by absorption, leaving the antitoxin intact. Since our experiments indicated that the protective property of our sera was not due to antitoxin, absorption experiments were made to find out whether the protective property could be separated from the antitoxin.

TABLE 4.

Agglutination of H. pertussis *Phase I by absorbed and non-absorbed antitoxins.*

	Serum dilutions								C
	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	
Antitoxin R28 absorbed (A)*	—	—	—	—	—	—	—	—	—
" " " (B)*	—	++	++	++	—	—	—	—	
" " non-absorbed	+++	+++	+++	+++	+++	+++	++	—	

*A—absorbed with two amounts of 0·3 gm. of living *H. pertussis*.
B— " " " " " 0·075 gm. " " " "

Two 10 ml. portions of antitoxin R28, diluted 1 in 2, were absorbed for 30 minutes at 45° C. with two successive lots of moist living organisms: portion A with 2 lots of 0·3 gm. of organisms and portion B with 2 lots of 0·075 gm. After absorption the organisms were removed by centrifuging and the sera heated to 56° C. for 30 minutes. The control non-absorbed antitoxin was heated to 45° and 56° with the absorbed sera.

In Table 4 the agglutination reactions of absorbed and non-absorbed antitoxins against a living suspension of a Phase I strain are given. Antitoxin titrations were made in mice (Table 5), and showed that absorption of the agglutinins did not affect the antitoxin content.

TABLE 5.

Titration in mice of absorbed and non-absorbed antitoxins.

	Quantity of serum in ml.			
	1/40 ml.	1/80	1/160	1/320
Toxin (4 M.L.D.) + Antitoxin R28 absorbed (A)*	888	888	+++	+++
" " + Antitoxin R28 absorbed (B)*	888	888	+++	+++
" " + Antitoxin R28 non-absorbed	888	888	+++	+++

* See footnote to Table 4.

The protective action of the absorbed antitoxin B was compared with that of the non-absorbed antitoxin, with results as given in Table 6. As in Table 3, the non-absorbed antitoxin again gave a protection which was statistically significant (as compared with the controls receiving no serum), whilst the absorbed serum gave no protection. The difference between the protective effect of the absorbed and non-absorbed antitoxins as shown by the proportion of mice with lungs clear of infection was also significant ($P = 0·034$) and this significance was reinforced by the difference found in intensity of lung infection in the two groups of mice concerned, although this last difference cannot be mathematically formulated. The results of this experiment show that the antitoxin, *per se*, has no power to control lung infection in mice.

TABLE 6.

Protective effect against intranasal infection in mice of absorbed and non-absorbed antitoxins.

Antitoxin given.	Mouse deaths.	Mouse lungs infected.	Intensity of lung infection.
Non-absorbed	0/13	7/13	3, 3, 2, 2, 1, 1, 1, 0, 0, 0, 0, 0, 0
Absorbed	0/13	12/13	4, 4, 3, 3, 3, 3, 2, 2, 1, 1, 1, 1, 0
Nil	0/13	13/13	4, 4, 4, 4, 4, 3, 3, 3, 3, 2, 2, 2, 2

The mice received 0·6 ml. of antitoxin diluted ½ and were given a small intranasal infecting dose (5,000 organisms) of *H. pertussis*. Survivors were killed 7 days later and cultures made of the lungs.

PASSIVE PROTECTION BY ANTITOXIN AGAINST *H. PERTUSSIS* GIVEN INTRAPERITONEALLY.

Bordet and Gengou (1909) noted a close similarity in the effects of living *H. pertussis* and of pertussis endotoxin, given intraperitoneally to guinea-pigs. They concluded that the animal receiving the living organisms was killed by a lethal dose of toxin and that no proliferation of the organism took place in the animal. Leslie and Gardner (1931) came to the same conclusions but noted that degraded strains of *H. pertussis* were less "toxic" than Phase I strains.

If death from *H. pertussis* given intraperitoneally were "purely toxic" then antitoxin should protect against such death, whilst sera containing no antitoxin should not. To test this point, 48-hour growths of *H. pertussis* on B.G. medium were suspended in saline and given intraperitoneally to mice. Serum doses were given by the same route 5–6 hours before the bacterial suspension. The M.L.D. of a culture was taken as the smallest dose which killed all the mice, using 4 to 6 mice on each dose. The M.L.D. of the cultures used throughout our experiments on the toxicity of *H. pertussis* given intraperitoneally, was in most instances 1 to 2×10^9 organisms, although occasionally it was as high as 4×10^9 and as low as 3×10^8.

Preliminary trials showed that the potent antitoxin R28 had a marked protective effect against living *H. pertussis* given intraperitoneally to mice, whilst the very weak antitoxin R19 had not. Attempts were made to obtain a quantitative comparison of the protective value of the five rabbit antitoxins by testing the action of graded doses of the antitoxins against a constant dose of about 4 M.L.D. of *H. pertussis*. No precise comparison was obtainable. All the sera of high antitoxin content afforded protection, whilst normal rabbit serum and human convalescent serum afforded none. Our results indicated that the number of M.L.D. of culture neutralized by 1 ml. of an antitoxin, fell as the number of M.L.D. in the test dose of culture was raised.

TABLE 7.

Protective effect of antitoxin and of convalescent serum against H. pertussis *given intraperitoneally.*

H. pertussis dose per mouse.	Serum dose per mouse. Nil.	AC8,0·3 ml.	Antitoxin, 75 units.
1×10^9	ssss	ssss	—
2×10^9	+2, +2, +2, +2	+2, +2, +2, +3	ssss
4×10^9	+1, +1, +1, +2	+1, +2, +2, +2	ssss
6×10^9	+1, +1, +1, +1	+1, +2, +2, +2	—
8×10^9	—	+1, +1, +1, +1	ssss
16×10^9	—		+3, +6, s s
32×10^9	—		+2, +2, +2, +3

s = mouse survived. +3 = mouse died 3 days after inoculation.

Table 7 shows the results of a comparison of the protective actions of a pooled antitoxin and the standard convalescent serum AC8 made by testing the effect of a constant dose of serum against graded doses of living organisms. The results showed marked protection by the antitoxin but none by the convalescent serum. Since the convalescent serum had been shown to have an antibacterial action both by its protective action against intranasal infection, and by *in vitro* tests, its complete failure to give protection in this experiment indicated that there was little or no proliferation of the organism in the peritoneal cavity of the mouse; whilst the marked protective action of the antitoxin indicated that the deaths in the experiment were primarily toxic.

H. pertussis was never recovered from heart's blood or lungs of mice protected by antitoxin against lethal intraperitoneal doses of the organism; but from those mice which received no serum, or not enough to protect them, the organism could always be recovered after death by culture of heart's blood or of the cut surface of the lung. The lungs of such mice however were not consolidated by an obvious pneumonia as is the case after intranasal infection, and the organisms cultured from such lungs were presumed to come from the blood circulating through the lung, not from the lung tissues.

EXTENT OF PROLIFERATION BY *H. PERTUSSIS* IN THE PERITONEAL CAVITY OF THE MOUSE.

(a) *Experiments without mucin.* The question as to whether any proliferation of *H. pertussis* occurs in the peritoneal cavity of the mouse was approached from another angle by seeking a method of sterilizating suspensions which would leave the toxin intact. If such a method could be found, proliferation would be revealed by a difference in the M.L.D. of the same suspension in the living and killed states.

Heating at 56° C. for six minutes killed *H. pertussis* but also destroyed the toxicity of toxin solutions and of bacterial suspensions. Heating at 45° C. for 30 minutes had no effect on toxin solution, but heating for four hours at 45° C. failed to sterilize living suspensions.

A concentration of 0·4 p.c. cresol was almost instantly lethal to *H. pertussis.* When part of a dense living suspension was treated with 0·4 p.c. cresol for one hour, centrifuged and resuspended in saline, the M.L.D. of the living suspension for mice was found to be 4×10^9 organisms and that of the cresol-killed suspension 10×10^9 organisms. Exposure of a toxin solution to 0·4 p.c. cresol for one hour by diluting the toxin with an equal volume of 0·8 p.c. cresol had the effect, however, of reducing both its lethal effect and its skin necrotic effect to less than one-tenth of their original values. Although the toxin in solution was destroyed with great rapidity by 0·4 p.c. cresol, the toxicity of intact *H. pertussis* was destroyed with comparative slowness by the same reagent. A suspension of *H. pertussis* kept in 0·4 p.c. cresol at 2° C. lost half of its toxicity in eleven weeks, whilst a suspension kept in 0·4 p.c. cresol at 37° lost three-quarters of its toxicity in five days.

Merthiolate added to dense suspensions of *H. pertussis* (50×10^9 per ml.) in concentration of 0·1 p.c., sterilized them in 1¼ hours at 37° C. provided the suspensions were thoroughly broken up. When part of a dense suspension was thus sterilized, the M.L.D. of the original living suspension for mice was found to be 4×10^9 organisms, whilst that of the merthiolate-killed portion was 6×10^9 organism. Treatment of a solution of toxin with 0·1 p.c. merthiolate at 37° C. for 1¼ hours reduced its toxicity to two-thirds of that of the untreated control, so that treatment with merthiolate caused the same proportionate diminution of toxicity in both toxin solutions and suspensions of living *H. pertussis.*

The results of the experiments on the effect of cresol on the toxicity of suspensions and of toxin solutions constitute further evidence that there was little or no proliferation of *H. pertussis* inoculated intraperitoneally into mice, whilst the more precise results obained with merthiolate indicate that no proliferation took place at all. There remains to be accounted for, however, the variation encountered in the M.L.D. of the living culture given intraperitoneally. This phenomenon may be explained as a variation in the toxin content of the organisms or as a variation in

ability of the culture to proliferate in the mouse. We have found, as did Evans (1940), that the yield of toxin from a number of different harvests of organisms has been constant, and it is to be noted that our experiments on the effect of cresol and merthiolate happened to be with suspensions of which the M.L.D. was the highest found in our work. The possibility that *H. pertussis* may under some circumstances multiply in the mouse peritoneum cannot therefore be ruled out, particularly in view of the results obtained by Mishulow *et al.* (1939).

(b) *Experiments with mucin.* Silverthorne (1938) and Miller and Silverberg (1939) in experiments on active immunity, used mucin as an adjuvant to reduce the number of *H. pertussis* required to kill mice by intraperitoneal inoculation. Their data indicate that the use of mucin effected only a slight reduction in the number of organisms required to cause death. Mishulow *et al.* (1939), using a slightly different mucin technique in passive immunity experiments, obtained a much greater reduction in the intraperitoneal killing dose of *H. pertussis* than did the previous workers.

Using the mucin technique of Miller and Castles (1936) and two different brands[1] of mucin, both of which had proved effective in work with the meningococcus, the M.L.D. of a suspension of *H. pertussis* was estimated when given intraperitoneally with and without mucin. In each case the mucin caused a twofold reduction in the number of organisms required to kill all the mice (from 2×10^9 to 1×10^9). When the technique described by Mishulow *et al.* (1939) was used for the preparation of mucin solution and bacterial suspension, the mucin again caused a twofold reduction in the number of organisms required to kill (from 4×10^9 to 2×10^9). The effect of the mucin on the killing power of the culture in these experiments was so slight that it is reasonable to doubt whether the effect was due to a proliferation of *H. pertussis* facilitated by the mucin, or to some other factor.

FURTHER EXPERIMENTS ON ACTIVE IMMUNITY TO TOXIN.

The possible presence of some degree of immunity to toxin was looked for in mice which had recovered from infection of the lung with *H. pertussis*. Three groups of mice, available as survivors from other immunity studies, were examined. These were (1) mice which received no preliminary immunization and recovered from intranasal infection; (2) mice which were immunized with a single dose of Phase I vaccine (1×10^9 organisms) and recovered from subsequent intranasal infection, and (3) mice recovered from intranasal infection and found totally immune to a second intranasal infection.

Using closely spaced toxin doses, no difference could be found between the M.L.D. of toxin for any of these groups of recovered mice, and the M.L.D. for control non-immunized mice.

Two groups of mice which had been immunized with three injections of cresolized toxin given intraperitoneally and subcutaneously respectively, were no more resistant to toxin than normal mice.

Attempts to immunize mice with sub-lethal doses of toxin by the subcutaneous, intraperitoneal and intravenous routes were not significantly successful. Some of the results obtained in these experiments paralleled those obtained by Wood (1940) with a toxin prepared by growing *H. pertussis* in peptone water plus rabbit serum. Mice which received $\frac{1}{8}$ to $\frac{2}{3}$ of an M.L.D. of toxin intravenously showed after 2–3 weeks the spleen and liver changes described by Wood. In addition the veins of such animals were so sclerosed and narrowed that it was difficult to give them a second intravenous injection. Mice which were given several sublethal doses of toxin intraperitoneally, all developed acute symptoms of shock after a subsequent intravenous dose and most of them died within a few hours of receiving the shock dose.

EFFECT OF TOXIN GIVEN INTRANASALLY.

Toxin given intranasally to mice in doses up to 10 M.L.D. in a volume of 0·05 ml. caused no obvious ill-health. When mice were killed 3 weeks after receiving 5 M.L.D. of toxin intranasally, sections of their lungs showed interstitial changes similar to those found by North and Anderson (1942) after intranasal inoculation of vaccine. In some of the lungs there were translucent yellowish patches such as have sometimes been observed in mice which have survived after intranasal infection with small doses of cultures of low grade infectivity. No organisms could be recovered from the lungs. Toxin heated at 56° for 30 minutes was found to produce the same cellular changes as did the unmodified toxin; hence the changes could not be attributed to the toxin *per se*. In later experiments a single intranasal dose of 3 M.L.D. of toxin caused no obvious macroscopic change in spleen or lung within 28 days. A single dose of 10 M.L.D. caused the appearance of translucent patches in the lungs of half the mice after 12 days, and after 28 days slight atrophy and paling of the spleen in half the mice together with enlargement of the lung in all of them. Sections of these enlarged lungs showed emphysema of varying degree together with peribronchial and interstitial infiltration, mainly by small round cells. Since intravenous injection of $\frac{1}{3}$ M.L.D. of toxin caused within 10 days marked paling of the spleen and shrinkage to half its normal volume, it follows that only a very small fraction of the toxin given intranasally was absorbed into the general circulation.

[1] Wilson 1701W and Armour.

In our general experience atrophy of the spleen has not been encountered in mice which have died after periods up to 28 days following intranasal infection. Splenic atrophy was specifically looked for in a series of mice infected with doses ranging in tenfold steps from 10^8 to 10^4 organisms. In this series deaths occurred up to 25 days and groups of survivors were killed and examined 16 to 28 days after infection. All the mice had normal spleens except some of the group of 11 survivors out of 18 mice infected with 10^6 organisms. When these 11 survivors were killed 26 days after infection, 7 of them had infected lungs and 5 had spleens with some degree of paling and of ½ to ⅔ normal volume.

To explain fluctuations which were encountered in the killing power of a constant dose of *H. pertussis* given intranasally we had examined washings from slope cultures and from suspensions, for "aggressive" properties. Aggressin-like properties were found in washings from cultures on B.G. medium but the results were not regularly reproducible. It seemed possible that the toxin might exert an aggressive action in the lung and so facilitate invasion by the organism. To test this possibility, groups of 5 mice were infected intranasally with small and large doses of *H. pertussis* suspended in 2½ M.L.D. of toxin in a volume of 0·05 ml., whilst control groups were given the same infecting doses in 0·05 ml. of saline. Toxin, given along with the infecting dose, caused no detectable increase either in the killing power of the infecting dose, or in the intensity of the infection produced by it.

DISCUSSION.

Although both active and passive immunity to lung infection by *H. pertussis* can be produced in mice, no evidence was found that pertussis endotoxin is concerned in such immunity. Neither animals immunized with vaccine or formolized toxin nor animals which had recovered from lung infection showed any greater resistance to toxin than normal mice. Passive immunity against lung infection is conferred by injection of antibacterial serum (North *et al.*, 1939) but not by antitoxin, since, although such immunity was produced by injecting antitoxic sera, the protective power of the serum was not related to its antitoxin content and was removed by an absorption (with bacteria) which left the antitoxin intact.

Our results have emphasized the difference in the nature of the deaths produced in mice by intranasal and by intraperitoneal inoculation of living *H. pertussis*. Death after intraperitoneal inoculation (which requires relatively large doses of organisms) is due to toxin and can be prevented by the use of antitoxin but not of antibacterial serum: death after intranasal infection with comparatively small doses of organisms is caused by an interstitial pneumonia, the course of which can be favourably influenced by antibacterial serum, but not by antitoxin *per se*. By laboratory procedure it is therefore possible to distinguish between antibacterial and antitoxic immunity. In the mouse, immunity to *H. pertussis* inoculated intranasally is essentially antibacterial; whilst immunity to *H. pertussis* inoculated intraperitoneally is essentially antitoxic, unless the technique used ensures that the inoculum contains distinctly less than 1 M.L.D. of toxin and that proliferation of the organism occurs in the animal. It is only when the technique conforms to these special conditions that the use of intraperitoneal inoculation of living *H. pertussis* as a test for antibacterial immunity becomes valid.

Wood (1940) found that mice which were given intraperitoneal doses of toxin acquired an increased tolerance to subsequent doses of *H. pertussis* given intraperitoneally, and concluded that such increased tolerance was not a manifestation of true immunity, but was due to local tissue changes produced in the peritoneum by the toxin. Our own experience leads us to support Wood's conclusion, and we wish further to point out that this apparent immunity may lead to fallacious results in experiments designed to demonstrate active immunity to pertussis by the method of immunizing mice with intraperitoneal injections of vaccine, and subsequently testing them for increased resistance to intraperitoneal inoculation of *H. pertussis*.

Toxin in the form of intact *H. pertussis* was destroyed very much more slowly by 0·4 p.c. cresol than toxin in solution. Since it is unlikely that so small a molecule as that of cresol cannot penetrate into the intact pertussis organism, it seems prob-

able that pertussis toxin does not exist in the intact organism as such, but in the form of some precursor not susceptible to rapid destruction by cresol. If such were the case it would then follow that the toxin is formed from some cellular constituent of the organism by a process which (1) occurs in the peritoneal cavity of animals, (2) occurs as an autolytic process when the cell is disrupted. The experience of Teissier *et al.* (1929) that injection of formolized intact *H. pertussis* into guinea-pigs provoked no antitoxin response, whereas injection of formolized toxin solution did so, lends support to the view that the toxin does not exist as such in the intact organism. Wood's contention that the toxin is a surface component of *H. pertussis* is, in our view, negatived by the fact that absorption of antitoxic serum with large quantities of *H. pertussis* removed no antitoxin, and by the relative slowness of the destruction of toxin in the intact organism by 0·4 p.c. cresol.

The inability of antitoxin to control the lung infection, contrasted with the ability of antibacterial serum to do so, raises a doubt as to whether the endotoxin is an important or significant factor in the pathology of the mouse lung infection. Such doubt is increased by the findings (1) that the toxin exerted no "aggressive" action in the lung, (2) that pertussis lung infection of long duration was rarely accompanied by the splenic atrophy characteristic of toxin absorption and (3) that toxin was not readily absorbed from the surface of the lung. A further implication of the last two findings is that in the pertussis lung infection any damage caused by toxin is likely to be found mainly in the lung itself.

SUMMARY.

Formolized pertussis toxin provoked an antitoxic response in rabbits but not in mice. No antitoxic response could be provoked in mice by injecting toxin or cresolized toxin.

The limited protection afforded by pertussis antitoxic serum against intranasal infection of mice by *H. pertussis* was not related to its antitoxin content but was due to antibacterial antibody present in the serum.

Pertussis antitoxin protected mice against the intraperitoneal inoculation of *H. pertussis* but antibacterial serum did not do so.

No proliferation of the organism could be detected after intraperitoneal inoculation of *H. pertussis* in mice. Mucin used as an adjuvant had a very slight effect on the killing power of a culture.

The effects of intranasal administration of toxin were studied. Given intranasally with *H. pertussis* the toxin exerted no "aggressive action".

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THE DETERMINATION OF SMALL AMOUNTS OF CARBON MONOXIDE IN AIR AND BLOOD

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The standard methods for the determination of carbon monoxide in low concentration are based on its oxidation by iodine pentoxide and the titration of the liberated iodine or by palladous chloride and the colorimetric determination of the reduced palladium. Their lack of specificity necessitates long trains of preliminary purifying vessels while large gas samples are required. Nothing better indicates the general lack of satisfaction with these methods than the host of modifications that have been proposed. Bland (1940), in this Institute, by the use of a catalytic method for the determination of minute amounts of iodine rendered the first method suitable for operation on a sample of a few hundred c.c. but the method is somewhat laborious and difficult, particularly for occasional use.

The delicate and specific reaction between carbon monoxide and haemoglobin has been little considered as a means for the determination of the former substance except in a few instances. The dissociation of carbon monoxide-haemoglobin (HbCO) is much affected by light, temperature, pH and above all the presence of oxygen. The pioneer and oft quoted work of Douglas, Haldane and Haldane (1912), whence they concluded that concentrations of oxygen up to 5–6 p.c. favoured the formation of HbCO when the haemoglobin was present in excess seems to need revision. It was based on an early and most difficult method for the determination of HbCO in the presence of HbO_2 and the experience gained during the experiments on which this paper is based lends no support to their view.

In 1912 Hartridge devised a reversion spectrometer for the determination of the relative proportions of HbCO and HbO_2 in mixtures of the two and used it for the study of some properties of the former substance. It is less widely known than it deserves. At present the writer's preference is for the original type with a diffraction grating and a direct scale for the arm which covers the HbCO–HbO_2 span. It is so simple as to be well within the improvisatory capacity of any worthy physical laboratory. With this apparatus the proportion of CO in air can be directly determined, provided that it is above 2×10^{-4}, by equilibrating in the dark with a small amount of dilute HbO_2 solution and measuring the position of the α band of the resultant mixture of HbO_2 and HbCO. Air containing more than about 3×10^{-3} of CO needs to be accurately diluted with air freed from CO with an iodine pentoxide furnace.

An attempt to use the formation of HbCO to determine smaller amounts of CO in air was made by Nicloux (1925). He claimed to have removed the oxygen completely from large volumes of air and to have determined the CO in the residual gas by a measurement of the volume necessary to convert about 30 p.c. of the Hb present in a bubbling tube to HbCO. He observed with a direct vision spectroscope only and a study of his published results and of the methods which he used is not very encouraging. The use of the so-called pyro-tannic acid method for this purpose does not appear to be very helpful. Its admitted specificity is insufficient to counterbalance its relative insensitivity.

Anson and Mirsky (1925) pointed out that in the absence of oxygen and at a low temperature the reaction between CO and Hb proceeds nearly to completion.

They used this fact to determine the degree of dissociation of carbon monoxide haemochromogen.

The methods to be described, here, while using the highly specific reaction between CO and Hb require only small samples of air and very simple apparatus. Their accuracy is sufficient for all determinations in connection with industrial hazards, public health, etc. They require little preparation for occasional determinations, are not unduly slow or laborious, and are not affected by most gaseous impurities.

The oxygen and carbon dioxide are absorbed by alkaline sodium hyposulphite solution preferably containing sodium anthroquinone β sulphonate (Fieser, 1924) which accelerates greatly the absorption of oxygen. The CO in the residual gas is absorbed by a solution of horse Hb of known oxygen capacity. Horse Hb was selected as the one most readily procurable commercially of those having a high affinity for CO and, its providential accompaniment, a broad "span" between the positions of the α bands of HbO_2 and HbCO. For a full discussion of this, see Barcroft, Anson, Mirsky and Oinuma (1925). The residual horse Hb is converted to HbO_2, the proportion of the two pigments determined with a Hartridge spectrometer and the CO content of the air sample calculated from the amount of HbCO found. The whole method was calibrated against samples of air containing known amounts of CO prepared by the dilution, with air freed from CO, of air containing large, known amounts of CO whose composition was determined with a Haldane gas analysis apparatus.

The equipment proposed for this method has been deliberately restricted to the utmost in order that it may be as widely available as possible. In particular, the use of rubber and of taps has been kept to a minimum. The problem of acquiring one good tap for each of the tonometers led to searches among old discarded apparatus; many contemporary machine-made taps are unreliable. Similar reasons led to the decision to exclude three-way taps from the absorption burette. The reagents need not be of high purity.

Since, however, very small amounts of CO are in question the utmost scrutiny must be directed towards any material which might conceivably absorb any CO only to release it at an inconvenient juncture. The apparatus and accessories should be kept and used where there is no danger of their becoming contaminated with the traces of coal gas which are apt to accumulate in ill-ventilated laboratories.

The distilled water should be fresh and of good quality. Unless a better still than the average laboratory one be available the small amounts needed for the haemoglobin solutions should be redistilled through a Kjeldahl spray top to avoid traces of Cu. The amount of haemoglobin in the blood may be determined by any of the standard methods available. The requirement is a self-consistent set of relative values. A glass (not a gelatine) colour standard in a colorimeter is as serviceable as any once it has been calibrated by the use of known gas samples in the apparatus itself. A series of determinations in which the Barcroft blood-gas apparatus was used to determine the haemoglobin showed that the method is so nearly quantitative as to provide a theoretical justification for its use. The dilute haemoglobin solution should have a pH of about 7·8 attained by the addition of small amounts of Na_2HPO_4. High salt concentrations are to be avoided. It should be stored in a good glass-stoppered bottle in the refrigerator and discarded at the end of the day. The rubber tubing should be impregnated with vaseline *in vacuo* at 80–90° C. For the taps a good rubber tap grease is necessary; costly lubricants such as "apiezon" are not. The rubber bands on the taps must be so arranged that any torsion tends to keep the tap turned shut.

The first apparatus is shown in Fig. 1. It consists of an absorption burette, some tonometers, an evacuable T piece and a mercury levelling bulb. About 150 c.c. of mercury are needed. The absorption burette (A) has a capacity of 110–120 c.c., not less. It is graduated at 15 c.c. from the top. Its lower end has a tube carefully rippled to take 5 mm. bore pressure tubing. At the top is a 1 mm. bore capillary tube bent as shown. Near the top is the filling orifice closed preferably with a hollow ground-glass stopper. If a rubber stopper is used instead the orifice must be slightly contracted immediately behind the lip to assist in maintaining the stopper securely in its place. The small bulb (B) in the capillary tube helps to ensure that no liquid from the absorption burette enters the tonometer. The tonometers (C) are each about 12 mm. bore and of about 11 c.c. capacity. The capacity of each must be accurately determined. The apparatus is fastened to a light wooden frame.

The performance of a determination involves, in addition to the final reading of the spectrometer, five operations: the preparation of the tonometer, the preparation of the absorption burette, the filling of the apparatus with the gas sample, the absorption of oxygen and the absorption of the carbon monoxide. The standard haemoglobin solution is made as follows: 2 c.c. of horse blood are diluted to 25·0 c.c. with distilled water. A suitable volume of this, usually 2·5–3·5 c.c. depending on its haemoglobin content, is measured into a 50 c.c. standard flask. 2·0 c.c. M/15 Na_2HPO_4 are added and the volume made up to the mark with distilled water.

Into a dry tonometer is measured accurately 0·1 times its volume of the standard HbO_2 solution. The tap, which must be clean and dry, is greased and inserted. The tonometer is evacuated with a good water-pump, being gently rocked to assist in the liberation of the oxygen. When the α band of the HbO_2 is no longer visible the tap is shut and the tonometer disconnected from the pump. A plug of cotton-wool is put carefully in its outlet, care being taken that it is not pushed down against the tap. The tonometer is then connected to the absorption burette. The latter is thus prepared: the mercury level is raised to about ⅔ the way up the burette and the clip (K, not illustrated) at the base of the burette is shut. Above the mercury is placed 10 c.c. of 2 p.c. commercial NaOH solution. In the hollow stopper, or in a small tube if a rubber stopper be used, are measured roughly 25–30 mg. of sodium anthroquinone β sulphonate and 0·3 gm. $Na_2S_2O_4$. If a hollow stopper holds the powders it is wiped, greased, inserted and secured with a rubber band. If a rubber stopper is used the burette is tilted in the left hand, the powders

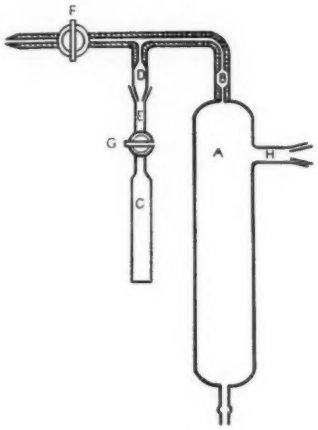

Fig. 1.

A Absorption burette, 110–120 c.c.
B Bubble trap, 0·5 c.c.
C Tonometer, about 11 c.c.
D 00 ground glass joint.
E Space filled with cotton wool plug.
F Burette tap, 1 mm.
G Tonometer tap, 2 mm.
H Filling orifice closed by hollow stopper, size ½.

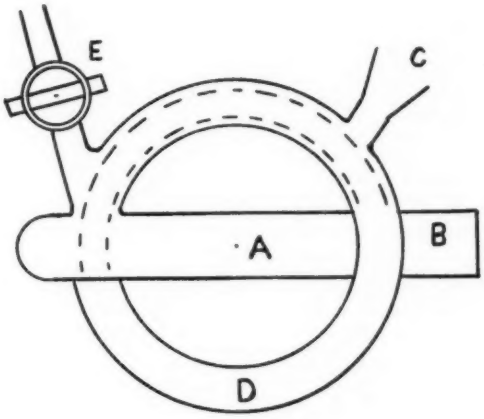

Fig. 2.

A Reading side 12 cm. long, 12 mm. bore.
B Reading section.
C Filling orifice closed by hollow glass stopper, size ½ or by rubber stopper.
D Absorption side, 8–10 mm. bore.
E Tap, 2–3 mm. bore.

The dotted lines indicate the continuation of the tube D to join the reading side near B. The mean diameter of the coil of D is about 75 cm.

tipped in and the stopper at once inserted and secured. The mercury is raised till the top of the aqueous layer is at the top of the burette. Clip (K) is shut. Then tap (F) is shut. Clip (K) is opened and the mercury lowered about an inch. Clip (K) is shut. The sample bottle and evacuable T piece are attached to tap (F). The T piece and absorption burette are evacuated with the water pump, filled with the air sample, re-evacuated and refilled with the air sample; the levelling bulb is then manipulated till the burette contains 15 c.c. of the air sample at atmospheric pressure. Tap (F) is shut and the T piece disconnected from the burette.

The oxygen is completely removed from the sample in the following way. The mercury is lowered to the bottom of (A) and clip (K) is shut. The supporting frame of the apparatus is inclined at about 60° to the vertical and it is gently shaken for 35 seconds. After being returned to its upright position and a pause of 10 seconds to allow the liquid to drain clip (K) is opened and the mercury level is alternatively raised and lowered slowly about 2–3 cm. to expel from the base of the capillary below (B) the small drop of solution which always accumulates there. No liquid must be allowed to pass (B). The mercury is then raised till the gas is at atmospheric pressure, the mercury is lowered to the bottom of (A), again raised as before and again lowered to the bottom of (A). After closing clip (K) the shaking, mixing, etc., are repeated 3 times, making 4 in all.

After the fourth shaking and the expulsion of the drop below (B) the tonometer tap (G) is opened and the mixing carried out to include the small amount of air remaining in the tonometer. The shakings, expulsion of the drop below (B), mixing of the air sample, including that in the tonometer, are done five times more, but taking 65 seconds for each shaking since allowance must be made not only for the rapid absorption of O_2 by $Na_2S_2O_4$ but also for the slower evolution of the last traces of O_2 from the haemoglobin solution. During the shakings the tonometer tap (G) must be shut. After the last shaking and a thorough mixing of the gas sample the tonometer is filled with the deoxygenated air sample to atmospheric pressure by manipulation of the mercury reservoir. If the dead space has been kept as small as possible the 15 c.c. is enough. After the tap has been closed the tonometer is detached from the burette and its wool plug removed. It is then rotated for two hours in the dark at 0° C. at about 80–100 r.p.m. with its axis horizontal. At the end of that time the tonometer is examined with the Hartridge spectrometer. Either haemoglobin or HbCO may be seen but no HbO_2. If any of the last-named is detected the determination must be discarded. If the inspection is satisfactory the tap is removed and a few c.c. of air blown in. After a few seconds rocking to convert the residual Hb to HbO_2 the position of the α band is determined with the spectrometer. From the amount of HbCO thus found the proportion of CO in the original sample may be calculated. The assumption is made that of the air a constant proportion, approximately 21 p.c., is absorbed by the alkaline $Na_2S_2O_4$.

The results quoted in this paper were based on HbO_2 solutions whose concentrations were determined with a Barcroft apparatus. They show how nearly theoretical is the method and what degree of consistency may be reasonably expected.

Fig. 2 shows a second apparatus based on the same essential principles, suitable for most samples of air except perhaps those containing large amounts of H_2S or SO_2. It uses rather larger samples of air than is practicable with the first apparatus on account of the heavy weight of mercury involved; this makes the reading somewhat easier. It consists essentially of a double spiral tube, with the free ends joined, fitted with a tap through which the HbO_2 solution and air sample are admitted and a hollow ground stopper in which are placed 0·3 gm. $Na_2S_2O_4$ and 30 mg. sodium anthroquinone β sulphonate. In the absorption side are placed 3·0 c.c. 6 p.c. NaOH. In the reading side is placed 1·0 c.c. of the HbO_2 solution, of about 0·15 p.c. in M/375 Na_2HPO_4, previously standardized. After tilting the salts into the NaOH solution the apparatus is gently rocked mechanically 80–100 times per minute for 3 hours at 0° C. in a horizontal path at right angles to the axis of the spiral. A displacement of 25 mm. on either side of the mean position is sufficient. For most determinations a calibrated shorter period of rocking is sufficiently accurate. The absorbing fluid acts as a crude pumping device for continually mixing the air sample and bringing fresh portions into contact with the haemoglobin solution which spreads in a fairly thin film over the larger bore tube (A) in the reading side. Equilibrium is surprisingly swift, the oxygen of the HbO_2 dissociating in less than 20 minutes, and most of the CO has combined with the Hb in 40 minutes. The readings with the spectrometer and the subsequent calculations are performed as in the first method with the exception that no allowance has to be made for the amount of O_2 absorbed. The volume of air is the volume of the apparatus, which must be accurately determined, less 4·0 c.c. for the volumes of the two solutions.

To avoid the use of mercury, a device was constructed for filling the spiral tonometers. The mercury reservoir and levelling bulb which are customary for such purposes were replaced by a selected glass syringe using medium oil as a lubricant. It was connected to a 3-way tap and a small manometer for reading the pressure. The manipulation is precisely the same as when the more usual mercury-operated reservoir is used.

The spiral tonometer can also be used very simply for the determination of CO in blood. In this case the removal of the oxygen is unnecessary owing to the relatively large amount of CO available. In the absorption side (D) are placed 1·0 c.c. of the blood under examination, 3 c.c. of 2 p.c. Na_2CO_3, enough solid saponin to haemolyze the cells and 0·3 c.c. of strong $K_3Fe(CN)_6$ to liberate the CO. The apparatus is rocked 80 times per minute in the dark for two hours at laboratory temperature. The CO is absorbed in the reading side in 1 c.c. of a dilute oxyhaemoglobin solution to an extent depending on the concentration of CO and on the relative affinities of CO and of O_2 for the haemoglobin used. Variations in the concentration of O_2 can usually be ignored. Calibration is by the use of known samples. The oxyhaemoglobin solution used is made from the blood under examination in the same way as when the apparatus is used for the determination of CO in air.

Though for exact work the oxyhaemoglobin content of the blood must also be known it is usually unnecessary for industrial purposes since symptoms of CO poisoning are accompanied by a much higher CO concentration than will be found in the blood of even a heavy smoker. The method is, however, so delicate that it will determine in a 2 c.c. sample of the blood of a non-smoker the small amount of CO that is always present.

While the measurement of the position of the α band with the Hartridge can quite well be carried out without removing the haemoglobin solution from the tonometer the accuracy is somewhat increased if it be transferred to a small rectangular cell for the spectrometry.

TABLE 1.

Determination of CO in known samples using the small tonometers. Haemoglobin solution standardized with Barcroft's apparatus.

Parts by volume × 10^6.	
Known.	Found.
54	55, 56, 53, 61
57	57, 55, 51, 52
10	9, 6, 8, 11, 9, 11, 10
49	42, 48, 45, 45
48	47, 48, 44, 49
Determinations using spiral tonometers.	
48	45, 44, 45

TABLE 2.

Determination of CO in human blood using spiral tonometers.

Volume of blood saturated with CO.	Volume of normal blood present.	P.c. span of Hartridge.
None	2 c.c.	14
None	1 c.c.	6
0·1 c.c.	0·9 c.c.	56
0·1 c.c.	None	45
0·1 c.c.	None	46
0·15 c.c.	None	60
0·15 c.c.	None	58
0·2 c.c.	None	65
0·25 c.c.	None	72

SUMMARY.

Two methods are described for the determination of carbon monoxide in samples of air of less than 25 c.c. The methods are based on the combination of CO with haemoglobin and are sensitive down to 1 part per 100,000.

The second apparatus is also suitable for the determination of CO in blood. It can determine the CO in 2 c.c. of the blood of a non-smoker and is sufficiently accurate for cases of suspected slight CO poisoning.

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ANTIBACTERIAL SUBSTANCES PRODUCED BY MOULDS

2. THE ANTIBACTERIAL SUBSTANCES PRODUCED BY SOME COMMON PENICILLIA.

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A previous paper (Atkinson, 1943) mentioned the frequency with which penicillia produce antibacterial substances. A new substance called penicidin was formed by one of the strains studied (No. 9) and the general properties of this substance have been described (Atkinson, 1942). The present work attempts to identify the antibacterial substances responsible for the activity of all the active penicillia so far isolated. These moulds were all naturally-occurring strains obtained from a variety of sources including fruit, vegetables and laboratory media. Of 68 penicillia tested, 18 have shown activity against various bacteria. These active moulds were divisible into two groups, Group 1 containing those acting mainly on Gram-positive bacteria and Group 2 those acting on both Gram-positive and Gram-negative bacteria. In Group 1 the active substance could be either penicillin (Fleming, 1929) or citrinin (Raistrick and Smith, 1941), and in Group 2 penicillic acid (Oxford, Raistrick and Smith, 1942) or penicidin, as any of these substances may be produced by a penicillium.

EXPERIMENTAL METHODS.

Modified Czapek-Dox medium as previously used (Atkinson, 1943) was employed throughout for the cultivation of the moulds. When the concentration of active substance was at its peak, the metabolism solution was removed from the mycelial felt and submitted to a series of tests for identification of the active substance. Some of the tests were applied to both Group 1 and Group 2 moulds while other tests were specifically designed for one group or the other. The tests applied to both groups were as follows:

(a) colour with $FeCl_3$, (b) effect on 1 p.c. $KMnO_4$, (c) effect on activity of low pH (produced with HCl or H_2SO_4), (d) effect on activity of high pH (produced with NaOH or KOH).

Special tests applied to Group 1 metabolism solutions were as follows:

(e) For the detection of penicillin the metabolism solution was adjusted to pH 2 and extracted immediately with ether. The ether extract was evaporated *in vacuo* at a temperature not exceeding 40° C., in the presence of one-quarter of its volume of water. The water, after evaporation of the ether, was then tested for activity against *Staph. aureus*. The extracted metabolism solution after neutralization was also tested for activity against *Staph. aureus* to detect any residual activity after the above treatment.

(f) For the detection of citrinin the metabolism solution, which should be orange-yellow, was strongly acidified with HCl when citrinin should be precipitated and the colour discharged. The yellow micro-crystalline precipitate of citrinin, separated after standing overnight at 2° C., was tested in aqueous solution for activity against *Staph. aureus*.

Special tests applied to Group 2 metabolism solutions were as follows:

(g) For the detection of penicidin the metabolism solution was adjusted to pH 7·4 and extracted with ether. The ether extract was shaken with dilute H_2SO_4 (pH 4) which, after separation from the ether, was neutralized, concentrated *in vacuo* and tested for activity against *Bact. typhosum*. The ether extract, after treatment with dilute H_2SO_4 was evaporated *in vacuo* in the presence of one-quarter of its volume of water; this, after removal of the ether, was tested for activity against *Bact. typhosum*. The activity of the extracted metabolism fluid was also tested in the same way.

[1] Part of the expenses of this work was provided by a grant to the Institute from the University of Adelaide.

In cases where the neutralized acid extract showed marked activity it was tested with $FeCl_3$, $KMnO_4$ and by the sodium nitro-prusside test, all of which may be used to identify penicidin. As all the moulds of Group 2 appeared to owe their activity to penicidin no further tests for penicillic acid were performed. It was deemed unlikely that this substance would be present in Czapek-Dox cultures as Oxford, Raistrick and Smith (1942) report that very little, if any, penicillic acid is produced on this medium, the best yields being obtained on Raulin-Thom medium.

RESULTS.

(a) With $FeCl_3$ no marked colour appeared.

(b) With neutral 1 p.c. $KMnO_4$ the metabolism solutions of Group 1 produced only slight decolourization in the cold whereas the metabolism solutions of Group 2 produced immediate decolourization.

(c) After 10 minutes at pH 2 the activity of Group 1 was destroyed but that of Group 2 was unaffected.

(d) After 10 minutes at a pH above 8 the activity of both Groups was destroyed.

(e) With Group 1 the extraction at pH 2 produced an active solution.

(f) The metabolism solutions of Group 1 were not orange-yellow and no precipitate appeared after strong acidification.

(g) The neutralized acid extracts of the metabolism solutions of Group 2 all showed activity. All rapidly decolourized neutral 1 p.c. $KMnO_4$ in the cold and those showing strong activity also gave a red colour (positive) in the sodium nitro-prusside test and a pale brownish-red colour with $FeCl_3$. Depending on the number of ether extractions the metabolism solutions after extraction exhibited varying degrees of activity. The ether extracts after washing three times with dilute H_2SO_4 were inactive, but in some cases some activity remained after washing only once.

From the results it appears that the commonly-occurring penicillia showing antibacterial action owe their activity to either penicillin (Group 1) or penicidin (Group 2). Some tests correlating the degree of activity of metabolism solutions of Group 2 with their ability to decolourize 1 p.c. $KMnO_4$ in the cold showed that the two tests are closely related. The greater the activity, the greater the volume of $KMnO_4$ decolourized by a given volume of metabolism solution. Thus the $KMnO_4$ test offers a rough but rapid qualitative and quantitative test for penicidin.

DISCUSSION.

It appears that antibacterial activity is frequently found among the penicillia. Here Group 1, which produced penicillin, consisted of common contaminants of laboratory media. Members of Group 2 which produced penicidin, were mainly found contaminating fruit and vegetables. It is interesting that moulds as ubiquitous as those of Group 2 should possess a potent antibacterial action but whether this action is present under natural condition of growth has not yet been investigated. Work on the effect of different environments on the production of penicidin is now being done.

SUMMARY.

The antibacterial substances of a group of active penicillia have been investigated.

The moulds could be divided into two groups of which Group 1 owed its activity to the production of penicillin and Group 2 to the production of penicidin.

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THE EPSILON TOXIN OF *CL. WELCHII* TYPE D.

1. PROTEOLYTIC CONVERSION OF ε PROTOTOXIN INTO ε TOXIN BY TRYPSIN AND OTHER PROTEASES

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Three distinct toxins have been recognized in cultures of *Cl. welchii* type D, namely, α and ε by Glenny *et al.* (1933) and θ by Turner and Eales (unpublished). When highly toxinogenic strains are grown in a suitable medium at 37° C., the haemolytic α and θ toxins reach maximal concentration after only a few hours and then begin to deteriorate, whereas ε toxin does not reach maximal concentration until 4 or 5 days. Therefore, to produce maximal yields of ε toxin, type D cultures are incubated for at least 4 days. The investigations of Nicholson (1934–35), Kellaway, Trethewie and Turner (1940a, 1940b) and Gordon, Stewart, Holman and Taylor (1940) support other evidence that the main clinical and pathological findings in enterotoxaemia of sheep and other herbivora are the result of ε intoxication. Although the disease often appears to develop very quickly, the ileum in fatal cases frequently contains ε toxin in concentration much higher than that usually attained in the most potent cultures in four days.

Bosworth and Glover (1934–35) set out to determine the reason for the higher toxicity of the bowel contents and made the important observation that culture filtrates or solutions of dried "toxin" of *Cl. welchii* type D acted upon by small quantities of trypsin became, after a short period, much more toxic. This phenomenon, which they referred to as the "activation of type D toxin", was prevented if the trypsin was previously treated with normal "antitryptic" serum. Crude trypsin, purified trypsin prepared by Northrop's method, and a "purified form of chymotrypsin" activated type-D toxin, but pepsin, papain, diastase and amylase did not. They apparently regarded activation as specific to the tryptic proteinases, and postulated that trypsin alters the ε toxin in such a manner as to form with trypsin a hypertoxic complex with some of the properties of histamine. In spite of the increased toxicity the combining power was not changed, and they considered this as "additional evidence that the results cannot be explained by the supposed presence of an inactive prototoxin which is converted into a 'D' type of toxin[1] by trypsin".

We have investigated the phenomenon in greater detail and in this paper show than many proteolytic enzymes besides trypsin are able to "activate" type-D toxin, and present evidence which strongly suggests that the phenomenon is the result of proteolytic conversion into ε toxin of an atoxic ε prototoxin with the same combining power as ε toxin.

TECHNIQUE.

Toxins were prepared by growing strains for 6 days at 37° C. in "V.F." broth with 0·25 p.c. glucose at pH 7·4 (referred to as "test medium"); this ensured maximal concentration of ε toxin but almost complete deterioration of α and θ. The bacterial cells were separated by strong centrifugation.

In reporting the titrations of toxins we use the term LD_{100} to indicate the smallest dose that killed within 48 hours both (i.e., 100 p.c.) of two mice injected intravenously. Usually the dilutions differed by 50 p.c., but occasionally we used 20 p.c. spacings. These crude end-points were

[1] i.e. the ε toxin.

sufficiently accurate for most of the work, but when greater accuracy was necessary the LD_{50}, the dose killing 50 p.c. of injected mice, was determined by injecting groups of 6 mice with doses at 10 p.c spacings, and then applying the probit method of Bliss (1938) as modified by Litchfield and Fertig (1941).

For routine tryptic activation, 1 p.c. of Difco Trypsin "1: 250" was added to toxin solutions without adjusting the pH, and after 45 minutes at 37° C. the necessary dilutions were made in normal saline and injected into mice; 1 p.c. is unnecessarily large but was convenient to weigh out when small volumes of toxin were used. If undiluted trypsinized toxin was to be injected, 0·25 p.c. of trypsin was used, 0·5 ml. ($\equiv$ 1·25 mg.) of which is well tolerated by mice.

We refer to the LD_{100} of untreated toxin as the primary LD_{100} to that of trypsin-activated toxin as the activated LD_{100}, and to the ratio of the primary LD_{100} to the activated LD_{100} as the activation ratio or A.R.

For much of the work on the mechanism of activation, two dried toxins were used. They were precipitated by saturating with ammonium sulphate 6-day cultures freed from their cells, and were dried *in vacuo* over sulphuric acid. Toxin "170 D" was further purified by Dann's method as described by Kellaway, Trethewie and Turner (1940a). Toxin "WD 8", a brownish powder, contains about 15 p.c. of ammonium sulphate; the primary LD_{100} is 0·0156 mg., the activated LD_{100} is 0·0039 mg. and the A.R. is therefore 4; the LD_{50} was determined as 0·00931 $\pm$ 0·00026 mg. Examined by the techniques described by van Heyningen (1941) it contains no α or θ toxin in 10 mg. Toxin "170 D" is a light greyish powder; the primary LD_{100} is 4·0 mg., the activated LD_{100} is 0·0312 mg., and the A.R. is 128; the activated LD_{50} was determined as 0·0197 $\pm$ 0·00003 mg. It contains no θ toxin in 10 mg., and 2·5 mg. gives only a faint reaction with lecithovitellin, corresponding to 1 mouse LD_{100} of α in about 226 mg. Both toxins may thus be regarded as free of α and θ toxins in the doses used.

Bromelin was prepared by the method of Chittenden (1894) except that acetone instead of alcohol was used as precipitant. Euphorbain was prepared by the method of Ellis and Lennox (1942). *Aspergillus flavus-oryzae* protease was prepared from cultures on bran medium.

In determining antitoxin combining power (C.P.) the standard of reference is a dried potent antiserum ("Sh. 1") prepared by Dr. D. T. Oxer, of the Commonwealth Serum Laboratories, Melbourne, and standardized by him at 220 arbitrary units[2] per ml. The L+ dose of toxin is taken as the amount which, when mixed with one unit of antitoxin, kills within 48 hours 50 p.c. of mice injected intravenously. Our standard toxin "WD 8" contains about 110 LD_{100} per L+ dose and hence fractional test doses, determined experimentally against corresponding amounts of antitoxin, as low as L+/40 may be used if necessary.

Two methods were used for estimating proteinase activity. In some experiments the simple method of Burdon and Lafferty (1936) was used but for the bulk of the work, especially with bacterial enzymes, we used the carboxyl titration method of Waldschmidt-Leitz (1924) as modified by Maschmann (1937), with gelatin as substrate. As bacterial enzymes, centrifuged culture supernatants were used without filtration. The usual digestion mixtures consisted of 8 p.c. solution of a special laboratory batch of gelatine (Davis Gelatine Co.), 5·0 ml.; enzyme solution, 0·5 to 2·0 ml.; activator or inhibitor solution, 1·0 ml.; and water to 10 ml. The gelatine, enzyme and activator or inhibitor solutions were each separately adjusted to the required pH with sodium hydroxide; no buffers were used except for the trypsin estimations at pH 8·9. The incubation period was from 20 to 24 hours at 40° C. for bacterial enzymes; shorter periods were used for other enzymes. Where cysteine was used as activator 0·1 p.c. was added and the mixtures were incubated under hydrogen to prevent oxidation. Toluene was used as preservative. Titrations were carried out in duplicate on 2·0 ml. samples and the results expressed as increase in ml. N/20 KOH.

ACTIVATION OF TYPE-D TOXIN BY TRYPSIN.

The Activation Ratio.

We readily confirmed the main observations of Bosworth and Glover and found that after a short exposure to the action of trypsin *Cl. welchii* type D toxins, practically free of α or θ, might kill with smaller doses or more quickly than before. However, we soon found that toxins differed in the extent to which toxicity increased and we therefore systematically determined on 35 strains what we refer to as the primary LD_{100}, activated LD_{100}, and activation ratio (A.R.). Many of the strains were freshly isolated from cases of enterotoxaemia, mostly in sheep, but occasionally in other animals, e.g. "205" (Patna goat), "230" (ox); some were laboratory strains isolated for various periods. The primary LD_{100} varied from 0·5 ml. to 0·0025 ml. but the activated LD_{100} usually lay within the relatively narrow zone 0·008–0·001 ml. Consequently, if the primary toxicity was low, the A.R. was usually high, and *vice versa*. Strain "234" was exceptional since, although the primary LD_{100} was high, the activated LD_{100} was also high and the A.R. was therefore low.

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[2] Dr. Oxer informs us that it is impossible to relate the new arbitrary unit with his original (Oxer, 1936); however, he considers that the new unit is probably only slightly greater than the original, which has been lost.

The A.R. in this series of strains ranged from 1 to 500. The characteristics of the toxin of these 35 strains are shown in Table 1.

TABLE 1.

Characteristics of the toxin of 35 strains of Cl. welchii *type D.*

Strain.	LD_{100} (ml.) Primary.	Activated.	A.R.
166	0·0025	0·0025	1
222	0·016	0·008	2
Werribee	0·005	0·00125	4
227	0·008	0·002	
W2/C.S.L.	0·01	0·0025	
223, 405	0·016	0·004	
234	0·125	0·031	
206, 208, 211, 217, 237, 239, 284, 326, W.2	0·016	0·002	8
Rose A1	0·02	0·0025	
212, 230, 241	0·031	0·004	
209, 210, W32	0·031	0·002	16
Lancefield	0·04	0·0025	
Rose A2	0·08	0·005	
Rose B1	0·04	0·00125	32
231	0·25	0·008	
O'Connor	0·125	0·002	64
269, W29	0·5	0·008	
205	0·25	0·002	128
173	0·5	0·0025	200
170D	0·5	0·00125	400
W41	0·5	0·001	500

Although the A.R. of individual strains was usually fairly constant when tested under fixed conditions, small fluctuations were found, even when the strain was grown in the one type of medium.

It is common experience that strains gradually tend to lose their toxinogenicity, i.e. the LD_{100} gradually increases. We have found that this change does not necessarily affect the activated LD_{100} and hence the A.R. gradually increases especially when it is originally quite low. We isolated one strain of A.R. = 1 and another of A.R. = 2, but the A.R. quickly changed to 4 and 8, respectively. Most strains when freshly isolated were moderately toxinogenic and the A.R. ranged from 4 to 16. Some strains, freshly isolated from highly toxic ileal contents of animals dead of enterotoxaemia, produced so little primary toxin in test medium as to be virtually atoxinogenic; yet the activated toxicity was high and therefore the A.R. was correspondingly very high.

In this paper we refer to strains at the extremes of the series as low activation ratio (L.A.R.) strains and high activation ratio (H.A.R.) strains, respectively.

We found, as did Bosworth and Glover, that type-B toxin may be activated by trypsin: a dried ammonium sulphate precipitated toxin prepared in the usual way from an 18-hour culture killed with one-quarter the dose after activation. On the other hand, trypsin did not activate type-A and type-C toxins.

Conditions which Influence Activation.

For many of the experiments on the mechanism of activation we used the purified toxin prepared from the H.A.R. strain "170 D". It has the advantage of being practically atoxic when not activated but is very toxic when fully activated and in these experiments amounts were commonly used which, though completely atoxic when untreated, were equivalent to many LD_{100} when fully activated. We refer to these amounts of untreated toxin as containing a corresponding number of "potential LD_{100}".

The pH of normal saline solutions of "170 D" toxin in the usual concentrations is 5·40 and that of 1 p.c. solutions of our sample of trypsin is 6·15; the pH of mixtures of equal volumes of toxin solution and trypsin solution is 5·60.

Hydrogen-ion concentration. The influence of pH was observed by determining the least time necessary to activate fully 10 potential LD_{100} when incubated at 37° C. in a total volume of 0·5 ml. with an amount of trypsin (0·078 mg.) previously found to be adequate at the pH of these mixtures (i.e. 5·6). Trypsin solutions and toxin solutions were each separately adjusted

to a series of pH values by adding predetermined amounts of N/100 or N/500 NaOH or HCl, in order to avoid using buffers. Equal volumes at each pH were mixed and incubated. At intervals for a period of 60 min., 1 ml. samples were removed, quickly brought to pH 7·0 and diluted to 10 ml. of which 0·5 ml., corresponding to 1 potential LD_{100}, was injected into pairs of mice. Death of both mice was taken as evidence of complete activation. The results, plotted in Fig. 1, show that activation becomes more rapid as the pH approaches the optimum for tryptic digestion.

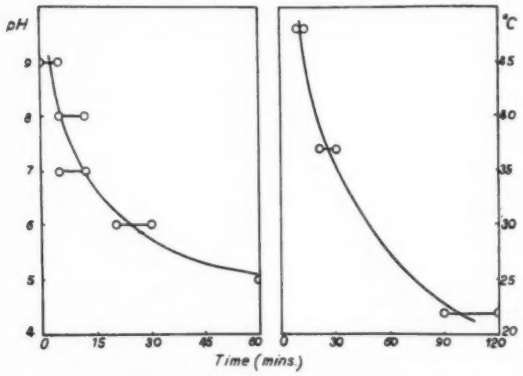

Fig. 1. (Left) Relationship between the time required for tryptic activation of "170 D" toxin and the pH, at 37° C. (Right) Relationship between time and temperature, at pH = 5·6. The bars indicate the time interval during which activation became apparent.

The pH of culture filtrates or supernatants in test medium ranges from 6·5 to 7·0 and is therefore not optimal. However, in practice and within limits this is not important provided adequate time, temperature and trypsin are allowed, and in all our experiments we have taken care that the conditions permitted complete activation.

Time and temperature. Solutions containing 2 potential LD_{100} of "170 D" toxin per ml. and the minimal amount of trypsin necessary for complete activation at 37° C. were incubated at 22° C., 37° C. and 48° C., respectively. At intervals, 0·5 ml. samples were withdrawn and injected intravenously into mice. The results are presented graphically in Fig. 1. At 37° C. toxicity had decreased somewhat after 4 hours and was quite absent after 18 hours, probably because of destructive digestion. Consequently, we adopted 45 minutes at 37° C. as adequate but not excessive in the pH range at which all our activation experiments were carried out, namely, 5·6–7·0.

Concentration of trypsin. At pH 5·6, the smallest amount of trypsin capable of completely activating 1 potential LD_{100} of "170 D" toxin in a volume of 0·5 ml. was determined within 20 p.c. as 0·03 mg., corresponding to a concentration of 0·006 p.c. The amount necessary to liberate at least 1 LD_{100} from 10 potential LD_{100} was 0·00014 mg. ($\equiv$ 0·000028 p.c.) and to activate completely 10 potential LD_{100} was 0·078 mg. ($\equiv$ 0·0156 p.c.).

For routine purposes, 0·25 p.c. of trypsin acting for 45 minutes at 37° C. can be relied upon to activate completely culture supernatants at their normal pH.

The Effect of Activation on the Killing Time.

We first determined the effect upon the killing time of increasing the number of lethal doses. Eight groups each of 6 mice were injected with doses of toxin "WD 8", ranging from 4 LD_{100} to 512 LD_{100}.[3] The results, set out in Table 2, show that, by increasing the dose of an untrypsinized potent toxin free of α and θ toxins, the killing time is progressively reduced to a few minutes, and also illustrate the very wide variation of killing time with each dosage.

TABLE 2.

Reduction of killing time with increased dosage.

Dose (LD_{100}).	Killing time (in minutes) Range.	Mean.	S.D.
4	75·0 –130·0	111·9	23·6
8	9·0 – 59·5	37·8	21·6
16	8·75– 25·5	19·9	7·0
32	4·5 – 16·25	10·8	4·5
64	5·5 – 12·25	9·5	2·7
128	5·25– 9·5	7·3	1·7
256	1·75– 7·0	5·0	2·3
512	2·0 – 4·5	2·8	0·9

[3] 512 LD_{100} = 7·98 mg., which contains only 1·2 mg. $(NH_4)_2SO_4$.

Excluding from the calculations the dose 4 LD_{100}, at which the relationship no longer holds, a simple parabolic relationship of the form $y = ax^b$ was shown to exist between killing time and dosage; all but one of the points fall within the error band $P = 0{\cdot}01$ (Fig. 2).

Since the m.l.d. of the toxins used by Bosworth and Glover was decreased about 30 or 40 times by trypsin, it seems likely that the reduction of killing time reported by them was due to the consequent 30- or 40-fold increase in the number of effective lethal doses.

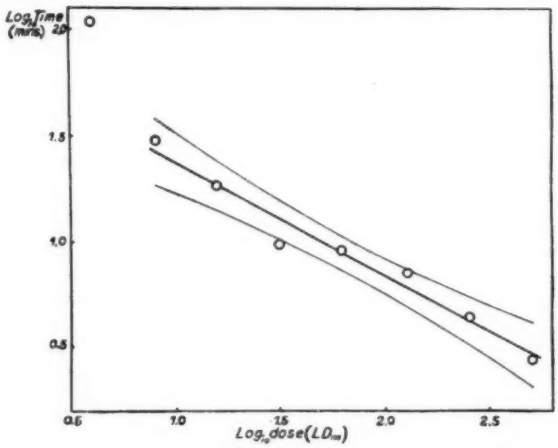

Fig. 2. Relationship between the killing time and the dose of "WD 8" toxin. Equation of the curve: $y = ax^b$, where $a = 82{\cdot}62$ and $b = -0{\cdot}5367$. $P = 0{\cdot}01$ that the true line lies between the limits of the two hyperbolae.

To determine whether trypsin-activated toxin kills more quickly than an equivalent number of lethal doses of unactivated toxin we compared the effects of injecting 16 LD_{50} of activated "170 D" toxin and of untreated "WD 8" toxin respectively into two groups of 20 mice. To activate "170 D" toxin the minimal amount of trypsin necessary for complete activation was used (0·025 p.c.) corresponding to 0·125 mg. of trypsin per dose. The killing time of 16 LD_{50} of activated "170 D" toxin ranged from 14 to 120 minutes (mean = 67·0) and of "WD 8" toxin, from 27 to 54 minutes (mean = 40·3). The difference, $26{\cdot}7 \pm 5{\cdot}74$, is highly significant and shows that the activated toxin actually killed a little less quickly than the untreated toxin.

To determine whether a greater amount of trypsin, not lethal in itself, might significantly decrease the killing time, we injected 20 mice with the same dose of "170 D" toxin activated with 10 times the amount of trypsin used before, i.e. 0·25 p.c., corresponding to 1·25 mg. per dose. The killing time[4] ranged from 11 to 102 minutes, and the mean (57 minutes) does not differ significantly from either of the others. In each case the range of killing time for trypsinized "170 D" toxin was greater than for "WD 8" toxin. The mode of death from trypsinized or untreated toxin did not differ in any characteristic way: after the injection the animals looked depressed and their breathing became deep and hurried. Some quickly developed severe inspiratory dyspnoea, exophthalmos and convulsions; they leaped into the air and soon died; this was usual when large doses of "WD 8" were given (25 or more LD_{100}). Those that died more slowly became cyanotic, the breathing became shallow and irregular and they died quietly. The signs thus resembled in some respects those of histamine poisoning, complicated by neurotoxic effects.

Inhibition of Tryptic Activation.

By normal serum containing "antitrypsin". It is important not to over-tax the "antitryptic" activity of the serum through using excessive amounts of trypsin. Our sample of serum was shown to be rich in "antitrypsin".

The minimal amount of trypsin capable of activating fully 10 potential LD_{100} of toxin "170 D" was dissolved in 0·1 ml. saline and left in contact with 0·6 ml. normal ox serum at room temperature for 30 minutes, after which 10 potential LD_{100} of toxin in 0·1 ml. saline was added and the mixture was incubated at 37° C. for 45 minutes. This was innocuous to mice. When saline was substituted for serum, injected mice died within three hours; the serum was therefore responsible for inhibiting activation. When toxin and trypsin were incubated at 37° C. for 45 minutes, the serum then added and incubation prolonged at room temperature for 30 minutes, injected mice died within 3 hours; this indicates that activation is not reversible by serum.

Heparin. Heparin has been shown to inhibit proteolytic activity of trypsin (Horwitt, 1940; Glazko and Ferguson, 1940). We found that heparin (250 units per dose), when substituted for ox serum in similar tests, behaved in a similar manner.

[4] Excluding one mouse that died in 3 minutes; it was probably hypersensitive to trypsin.

Egg-white antitrypsin. Egg-white antitrypsin prepared by the method of Balls and Swenson (1934) was shown to inhibit gelatin digestion and tryptic activation of toxin.

Thus three substances shown to inhibit the proteolytic activity of trypsin also inhibited the tryptic activation of toxin.

The Effect of Tryptic Activation upon Combining Power.

To avoid destructive tryptic digestion of the combining groups of the toxin or of the specific globulin, we used the minimal amount of trypsin, allowed the trypsin to act for a shorter period (20 minutes) than optimal, and then "neutralized" the trypsin by adding a known excess of normal ox serum rich in "antitrypsin". Dilutions, differing by 10 p.c., of unactivated and activated "170 D" toxin respectively were then incubated with 1/10 unit of antitoxin for 30 minutes and the L +/20 dose of standard toxin was added; incubation was continued for a further 30 minutes and the mixtures were injected into groups of 3 mice. In each case the L +/20 dose was found to be 0·33 mg., although toxicity was increased about 100 times after activation.

Relative Thermostability of Unactivated and Activated Toxin.

We used normal saline solutions of the widely different toxins "170 D" (A.R. = 128) and "WD 8" (A.R. = 4), of pH 5·60 and 5·55, respectively. Heat resistance was tested by exposing the solutions to 70° C. for 15 minutes.

The primary toxicity of "170 D" was only halved by heating, whereas that of "WD 8" was decreased more than 500-fold; on the other hand, if "170 D" were activated before heating, it behaved like "WD 8". Furthermore, heated "170 D" could still be considerably activated (toxicity one-fourth that of activated unheated toxin) whereas "WD 8" had been completely destroyed by heating and could not then be activated.

It is obvious that our results with "170 D" toxin do not support the statement of Bosworth and Glover that activated toxin heated at 70° C. for 15 minutes loses its increased toxicity and behaves like an equivalent amount of toxin heated in a similar manner. As "WD 8" toxin, whether activated or not, was completely destroyed by heating, the results obtained with it did not prove helpful in this regard.

It may be concluded that H.A.R. toxins are much more thermostable than L.A.R. toxins, but when activated behave like L.A.R. toxins.

ACTIVATION OF TYPE-D TOXIN BY OTHER PROTEASES.

All samples of unpurified trypsin at our disposal activated "170 D" toxin; on the other hand, a 20 p.c. extract of pig's intestinal mucosa in 87 p.c. glycerol, which hydrolyzed Witte peptone strongly but gelatine very slightly, was inactive. From this it appears that the intestinal "ereptic" enzymes (peptidases) are unable to activate type-D toxin.

In order to determine if trypsin is unique in this respect we examined several other proteases and found that many shared this property. Wherever activation is recorded, we have shown that the toxicity was specifically neutralized by anti-D serum and that activation and proteolytic activity were inhibited by normal ox serum.

Plant Proteases.

Papain. Very slight activation occurred after 18 hours at 37° C. under toluene with 1 p.c. papain (Parke, Davis & Co.) but, when 0·1 p.c. neutralized cysteine was added and incubation carried out anaerobically, activation was complete within 2 hours.

Maschmann (1938) claimed that normal "antitryptic" serum does not inhibit gelatin digestion by papain-cysteine.

However, the following results show, in increase of ml. N/20 KOH, the enhancing effect of cysteine (0·1 p.c.) upon gelatin digestion by this sample of papain (0·01 p.c.) and the inhibition of papain-cysteine by normal ox serum (10 p.c.).

	Incubation period.		
	1 hr.	2 hr.	4 hr.
No cysteine	0·00	0·01	0·02
Cysteine	0·11	0·19	0·31
Cysteine + normal serum	—	—	0·01

The extreme inactivity of some samples of papain, unless activated by cysteine, towards both "170 D" toxin and gelatin may explain the failure of Bosworth and Glover to obtain activation of toxin by papain.

Bromelin. A 1 p.c. solution of bromelin did not activate toxin even after 18 hours at 37° C. and had no detectable gelatin-digesting activity. However, like papain, when treated with cysteine it activated "170 D" toxin completely within 2 hours, and digested gelatin.

Euphorbain. A 0·5 p.c. solution of euphorbain (Ellis and Lennox, 1942) activated "170 D" toxin within 16 hours at 37° C.

Animal Proteases.

Pepsin. Taylor and Stewart (1941) reported slight activation of type-D toxin by pepsin, but suggested that it might have been due to contamination with trypsin. In our experiments pepsin did not activate "170 D" toxin at pH 7·0. We therefore adjusted the pH nearer to the optimal for pepsin, namely, to 4·19 after finding that L.A.R. toxin "WD 8" was not destroyed at this pH even after 20 hours at 37° C. However, a 1 p.c. solution of pepsin scales (Merck) failed to activate 10 potential LD_{100} of "170 D" even after 20 hours at 37° C. and pH 4·19. As judged by the Burdon and Lafferty technique the gelatine-digesting activity of pepsin at pH 4·19 was equivalent to that of 0·04 p.c. of trypsin at pH 5·6, which is the pH of mixtures of trypsin and "170 D" toxin. Since at this pH 0·0156 p.c. of trypsin was sufficient to activate completely 10 potential LD_{100} of "170 D" toxin, it is probable that pepsin is intrinsically unable to activate type-D toxin.

Lucilia cuprina *protease.* An actively proteolytic preparation from gut washings of larvae of the sheep blow-fly (*Lucilia cuprina*), reared aseptically on enriched artificial medium (Lennox, 1939), and a preparation from the liquefied medium in which they had grown, both activated completely a solution of "170 D" toxin within 30 minutes at 37° C.

Microbial Proteases.

Yeast protease. Autolysate of brewer's yeast was prepared by the method of Dernby (1917) and dialyzed for 4 days against distilled water. The gelatin-digesting activity at pH 7·0 was equivalent to that of 0·25 mg. trypsin per ml. at pH 5·6. Activation of "170 D" toxin occurred in less than 30 minutes at 37° C.

Mould protease. A highly proteolytic aqueous extract of the mould *Aspergillus flavus-oryzae* completely activated a solution of "170 D" toxin within 2 hours at 37° C.

Cl. histolyticum *and* Cl. sporogenes *proteases.* Two smooth strains of *Cl. histolyticum* and three strains of *Cl. sporogenes* were grown for 48 hours in V.F. broth. Gelatin-digesting activity of the germ-free supernatants of the two species was equivalent to that of 8·0 and 4·0 mg. respectively of trypsin per ml. at pH 5·6. Each supernatant, mixed with 20 potential LD_{100} of "170 D" toxin per ml., was incubated under toluene at 37° C. Before injecting mice with *Cl. histolyticum* mixtures, specific antiserum was added to neutralize its toxin. With all preparations, activation became measurable after 4 hours and was complete after 21 hours.

Cl. welchii *type A protease.* There are difficulties in demonstrating directly that proteases of *Cl. welchii* type D can activate type-D toxin, although there is ample indirect evidence, as will be shown in a subsequent paper; but it was not difficult to show that the proteases of type-A can activate "170 D" toxin.

A dried ammonium sulphate precipitate of culture filtrate of *Cl. welchii* type A, strain "Mulford", was shown by carboxyl titration to possess high proteinase activity, not enhanced by cysteine, against gelatine: 10 mg. increased the titre by 1·25 ml. N/20 KOH. A solution containing 10 mg. per ml. of this preparation and 8 mg. per ml. of "170 D" toxin was adjusted to pH 7·0 and cysteine 0·1 p.c. added. After 3 days at 37° C. anaerobically under toluene, anti-A serum was added to neutralize type-A toxin. It was found that the primary LD_{100} of "170 D" toxin had decreased from 4 mg. to 0·25 mg., and the A.R. from 128 to 8.

A 16-hour culture of *Cl. welchii* type A strain "247" in test medium was similarly shown to possess high proteinase activity which was increased about 100 p.c. by cysteine. In this supernatant "170 D" toxin was dissolved at the rate of 4 mg. per ml. and the solution incubated anaerobically under toluene at 37° C. for 5 days. It was found that the "170 D" toxin had become completely activated.

To summarize, toxin was activated by trypsin, papain-cysteine, bromelin-cysteine, euphorbain, and proteolytic preparations from *Lucilia cuprina,* brewer's yeast, *A. flavus-oryzae, Cl. sporogenes, Cl. histolyticum* and *Cl. welchii* type A; in each case (except *Cl. welchii* type A, in which it was not attempted) both toxin activation and proteinase activity were inhibited by normal "antitryptic" serum.

DISCUSSION.

Although confirming many of the observations of Bosworth and Glover, our evidence does not support the hypothesis by which they sought to explain the activation of type-D toxin by trypsin, namely, that trypsin forms with ϵ toxin a highly toxic complex with some of the properties of histamine but "so closely linked to the toxin molecule that specific antitoxin still affords complete protection".

From the stage when we found that the A.R. can vary enormously according to strain whereas the activated toxicity falls within relatively narrow limits, it became probable that nearly all samples of type-D toxin contain, besides ϵ toxin, an almost atoxic substance or prototoxin which occurs in variable proportions and may be converted into ϵ toxin by trypsin. The similar thermolability of activated

H.A.R. toxin and unactivated L.A.R. toxin supports this. The effects of pH, temperature, time and concentration of trypsin upon the progress and extent of activation are what would be expected if activation is a proteolytic phenomenon. Our demonstration that a large number of proteolytic enzymes from the most varied sources can activate toxin makes this even more probable.

Bosworth and Glover regarded their important finding, that after activation the C.P. of "toxin" remains unaltered in spite of increased toxicity, as strong evidence that activation cannot be explained as the conversion of a hypothetical prototoxin into toxin; however, this is equally consistent with our hypothesis, if we assume that prototoxin has the same C.P. as the toxin derived from it.

They laid considerable stress on the great reduction in killing time after activation as evidence that a histamine-like complex of toxin and trypsin, thus fundamentally different from ϵ toxin, is formed. This is not supported by our demonstration that trypsinized toxin does not kill more quickly than the same number of lethal doses of untrypsinized toxin, that the killing time decreases logarithmically as the dose of ϵ toxin increases arithmetically, and that appropriate doses of either untrypsinized or trypsinized ϵ toxin kill quickly with some of the signs of acute histamine poisoning. Indeed, since both ϵ toxin (Kellaway, Trethewie and Turner, 1940b) and trypsin (Rocha e Silva, 1938) release histamine from the tissues it is not surprising that the animals injected with either activated or unactivated toxin may show signs of histamine poisoning. For the same reasons, it might not have been surprising if ϵ toxin and trypsin, acting synergically, had killed more quickly than the same number of lethal doses of toxin alone, but in our experiments the killing time was slightly increased. This may be due to significant errors in the determination of the LD_{50}, which after all can never be determined with absolute accuracy; on the other hand, pharmacologically active impurities (Kellaway, Trethewie and Turner, 1940a) in "WD 8" toxin may have significantly reduced the killing time as compared with the purified "170 D" toxin. Trypsin greatly increased the variation in killing time and some mice died much quicker than the most susceptible mice in the group receiving "WD 8" toxin. Since Bosworth and Glover used only pairs of mice it is possible that by chance they used some mice especially sensitive to trypsin.

All the evidence strongly suggests that cultures of most strains of *Cl. welchii* type D contain, in addition to ϵ toxin, an almost atoxic relatively thermostable ϵ prototoxin with the same C.P. as toxin, the relative proportions in which these occur varying according to the strain, and that the "activation of type-D toxin" by trypsin, as described by Bosworth and Glover, is the result of proteolytic conversion of prototoxin into ϵ toxin, and not of ϵ toxin into a hypertoxic complex with trypsin. It is a simple extension of this hypothesis to assume that in type-D cultures ϵ toxin develops from prototoxin through the proteolytic action of intrinsic protease, that except for very rare strains this process is usually incomplete, and that many extrinsic proteases, including trypsin are able to complete the conversion under appropriate conditions.

We may regard ϵ prototoxin as an antigenic substance which is normally excreted by *Cl. welchii* type D and which, through the proteolytic loss of certain groupings from the molecule, becomes toxin. The nature of these masking groups has not been determined and indeed cannot be determined without the aid of highly purified prototoxin and enzymes. However, some hints are yielded by the studies of Bergmann and his associates on the specificity of proteinases, as a result of which it is now known that each proteolytic enzyme requires the presence of certain atomic groupings within the so-called backbone and within the side chain of the substrate molecule. The finding that both papain-cysteine and trypsin convert prototoxin, whereas pepsin does not, may indicate the presence of arginine or lysine residues as side groups and the absence of tyrosine or phenylalanine residues (Bergmann,

1942). The finding of Bosworth and Glover that "a purified form of chymotrypsin" was effective should be checked with chymotrypsin of undoubted purity, for it and pepsin have the same side-chain specificities, though different backbone specificities (Bergmann and Fruton, 1941). It is of interest that although chymotrypsin, unlike trypsin, is not inhibited by heparin (Horwitt, 1940), we found that heparin inhibited the gelatinolytic and activating effects of our sample of crude trypsin; this may indicate that chymotrypsin is unable to attack prototoxin.

In the second paper of this series (Turner and Rodwell, 1943) we bring forward evidence to support our hypothesis that ε toxin is derived by proteolytic degradation from prototoxin.

SUMMARY.

Activation of the toxin of *Cl. welchii* type D, as described by Bosworth and Glover, is not confined to trypsin but may be elicited by a wide variety of proteolytic enzymes.

The evidence strongly suggests that most type-D toxins contain, in addition to ε toxin, a relatively atoxic ε prototoxin with the same combining power as ε toxin and that activation is the result of proteolytic conversion of prototoxin into toxin.

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THE EPSILON TOXIN OF *CL. WELCHII* TYPE D

2. MECHANISM OF ITS DEVELOPMENT IN CULTURES THROUGH THE ACTION OF EXTRACELLULAR PROTEINASES UPON ε PROTOTOXIN.

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We have already presented evidence (Turner and Rodwell, 1943) that cultures of *Cl. welchii* type D which have been incubated for the optimal period (4–6 days) contain, in addition to ε toxin, an atoxic relatively thermostable ε prototoxin with the same combining power as ε toxin and that the relative proportions in which toxin and prototoxin occur in such cultures vary from strain to strain. It was shown that many extrinsic proteolytic enzymes, including trypsin, may convert prototoxin into toxin and it was suggested that ε toxin is produced during incubation of cultures through the conversion of a prototoxin by means of intrinsic proteases.

In this communication we bring forward further evidence which supports this hypothesis. We show that ε toxin develops from an ε prototoxin, which is antigenically complete but is atoxic; that prototoxin is excreted together with proteinases during the growth phase; and that the extracellular proteinases gradually convert prototoxin into toxin to an extent which is rarely complete and depends mainly upon proteinase concentration.

TECHNIQUE.

Preparation of toxins, their titration and tryptic activation, the general methods for proteinase estimation and the unit of antitoxin have already been described. The terms activation ratio (A.R.), low activation ratio (L.A.R.), high activation ratio (H.A.R.), primary LD_{100} and activated LD_{100} have been defined (Turner and Rodwell, 1943). The term "potential toxicity" refers to the toxicity after complete enzymatic activation, for which purpose we used trypsin. Potential toxicity can be expressed in terms of the number of lethal doses per ml.

For the estimation of dipeptidase and aminopolypeptidase, M/15 dl-alanyl glycine (dl-AG) and M/30 diglycyl glycine (G.G.G.) respectively were used, together with divalent iron as M/500 or M/1,000 $FeSO_4$ and 0·1 p.c. cysteine.

In the preparation of the proteases of *Cl. welchii* type D the lag phase of growth was reduced to a minimum by sowing rapidly growing cultures a few hours old into recently boiled medium cooled to 37° C.

To determine the combining power (C.P.) of toxins or toxoids the method described by Sterne and Mason (1941) was adapted. The titrations were in some cases determined at 10 p.c. spacings on groups of three mice. The number of units of antitoxin which would be bound by 1 ml. of the toxin or toxoid solution, assuming that the "law of multiples" held, was calculated and expressed as antigenic units (A.U.) per ml.

To determine antitoxin in serum samples the method described by Oxer (1936) was adapted: with our toxin as little as 1/24 U. per ml. could be detected.

THE EXTRACELLULAR PROTEINASES OF *CL. WELCHII* TYPE D.

We have suggested the possibility that, in cultures of type D, intrinsic proteolytic enzymes convert ε prototoxin into ε toxin. Since no information was available in the literature concerning the proteases of type D, it was necessary to study certain aspects relevant to our investigation of the mechanism of the activation of toxin.

Weil, Kocholaty and Smith (1939) found that 18- and 24-hour filtrates of *Cl. welchii* type A are practically free from peptidases and that increase in amino nitrogen, with gelatine as substrate, is attributable to proteinase action alone. To determine if peptidases are excreted by young cultures of type D, cell-free supernatants of 6- and 16-hour cultures of the toxinogenic strains "326" and "223", and of 5-day culture of "223", were examined for dipeptidase and aminopolypeptidase activity. Cysteine and divalent iron were used as activators (Maschmann, 1938d).

One ml. of supernatant was used in the digestion mixtures; incubation period 18 hours at 40° C.; pH = 7·8 for dl-Ag and 7·2 for G.G.G. The results (ml. N/20 KOH) were as follows:

Strain.	Age of culture (hours).	Substrate. dl-AG.	G.G.G.
326	6	0·05	0·05
326	16	0·07	0·07
223	6	0·04	— 0·01
223	16	— 0·02	0·01
223	120	0·20	0·47
223	120 (no activators)	— 0·01	— 0·01

We felt justified in assuming that supernatants of young cultures of type D contain only insignificant amounts of peptidase and that increase in carboxyl titration, with gelatine as substrate, is a measure of proteinase activity. As subsequent work showed that only the proteinases are concerned in the development of toxin, attention was thereafter confined to them.

Weil, Kochalaty and Smith (1939) considered that *Cl. welchii* type A secretes only one proteinase, which is activated by cysteine and still more by cysteine and divalent iron. Smith and Lindsley (1939) and Smith (1940) found that the gelatin digesting activity was not inhibited by normal serum. On the other hand, Maschmann (1938a, b, c, d) considered that it produces two distinct proteinases, referred to as anaerobiase and collagenase respectively, both of which have a pH optimum of 7·0. Anaerobiase, which is typically intracellular but often appears in the culture fluid quite early, is activated by cysteine but is inhibited by iodoacetic acid, attacks clupeine as well as gelatine, and is inhibited by normal serum; collagenase, which is the typical extracellular proteinase, is not activated by cysteine nor inhibited by iodoacetic acid, attacks gelatine but not clupeine, and it is not inhibited by normal serum, but by specific anti-collagenase which occurs in anti-A sera.

With these findings in mind we examined the proteinase system of *Cl. welchii* type D and the results were as follows:

pH Optimum.

The gelatine solution was adjusted to give the required range of pH values, and the pH of the digestion mixtures was then stabilized by means of acetate-veronal-HCl buffer mixtures (Michaelis, 1931). Digestion mixture: 8 p.c. gelatine solution, 5·0 ml.; 16-hour supernatant of strain "326", 0·5 ml.; 1·0 p.c. cysteine, 1·0 ml.; buffer 3·0 ml.; water to 10 ml. Toluene. H_2. Temperature 40° C. Digestion period 21 hours.

Fig. 1 shows that the optimal pH lies very close to 7·0. It is important to note that in our medium the pH of all strains, after 24 hours' growth usually lies between 6·5 and 7·0, which is the relatively flat region of the activity curve.

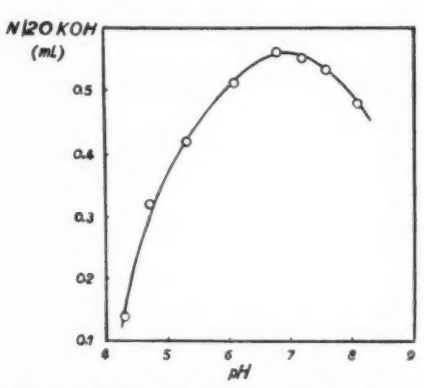

Fig. 1. pH activity curve of extracellular proteinase system of 16 hour cell-free supernatant of *Cl. welchii* type D, strain "326".

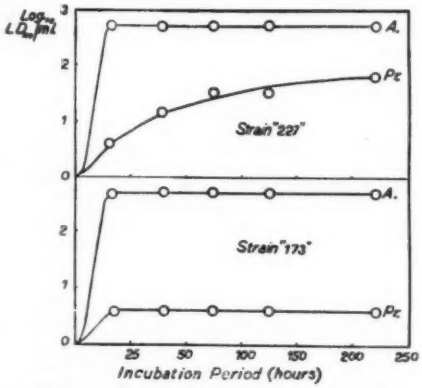

Fig. 2. Relationship between the primary toxicity (Pr.) and potential toxicity (A) of the L.A.R. strain "227" and the H.A.R. strain "173" respectively, at various stages of incubation.

Rate of Appearance of Proteinases.

A rapidly growing culture of strain "223" was sown into the test medium. On samples removed at intervals and immediately chilled, direct cell counts were made and the remainder strongly centrifuged at 4·5° C. Proteinase activity of 2 ml. samples of the cell-free supernatants was estimated in the usual manner at pH 7·0 with and without cysteine. Cell count and extracellular proteinase, with or without cysteine, both reached maxima at about 6 hours.

Effect of Activators and Inhibitors.

Cysteine, iron and iodoacetic acid. We examined 6-hour supernatants from 23 strains of which the A.R. varied from 4 to 128. Digestion mixtures were incubated both with and without cysteine for 24 hours. Five H.A.R. strains had little or no proteinase activity, either with or without cysteine. The proteinase activity, without cysteine, of the other strains of which the A.R. ranged from 4 to 64, corresponded to an increase of from 0·38 to 1·13 ml. N/20 KOH, but the percentage activation ranged from 11 to 148.

In these experiments we used 2 ml. samples of the supernatants. However, we later found that with the most active strains the relationship between amount of supernatant and the increase in titre appreciably deviated from linearity if amounts of supernatant greater than 0·5 ml. were used and therefore in subsequent tests the amount was reduced to 0·5 ml.

To examine the effects of iron, cysteine and iodoacetic acid we used 0·5 ml. amounts of two 16-hour supernatants of the L.A.R. strains "227" and "326", and compared the effects of cysteine, of cysteine + divalent iron, of iodoacetic acid (M/500), and of cysteine + iodoacetic acid. The results are summarized as follows:

Activator or inhibitor.	Strain. "227"	Strain. "326"
None	0·08	0·26
Cysteine	0·19	0·50
Cysteine + iron	0·08	0·32
Iodoacetic acid	0·09	0·27
Cysteine + iodoacetic acid	0·08	0·27

Thus cysteine greatly increased proteinase activity, but both divalent iron and iodoacetic acid abolished the increase. Iodoacetic acid did not inhibit initial proteinase activity. We decided that the addition of iron was contra-indicated.

Normal "antitryptic" and specific antisera. Using 5-hour and 15-hour supernatants of the L.A.R. strains "223" and "326", and cysteine as activator, we compared the inhibiting effects of the following sera against type-D proteinases: normal horse; normal ox; anti-*Cl. welchii* A (horse) serum containing 100 anti-α units per ml.; anti-*Cl. welchii* D (goat) serum prepared from the L.A.R. strains "166" and containing 190 anti-ε units per ml.; anti-*Cl. welchii* D (sheep) serum, prepared from the low-proteinase-producing H.A.R. strain "170 D" and containing 125 anti-ε units per ml.

All the *Cl. welchii* antisera inhibited strongly (54–68 p.c.) in the doses used, except the anti-D serum "170 D", which was without effect. The two normal sera produced less inhibition (16 and 30 p.c., respectively). Increasing the serum in the digestion mixtures from 0·06 ml. to 0·5 ml. did not alter the degree of inhibition by normal ox serum or by anti-D "166" serum (27 and 68 p.c. respectively); apparently even the lowest concentration of the sera (0·6 p.c.) was above the range in which a linear relationship between serum concentration and inhibition might be expected.

These results are consistent with Maschmann's hypothesis that anti-*Welchii* sera contains, in addition to the non-specific inhibitor against anaerobiase, a specific anti-collagenase, if we assume further that type-D collagenase is neutralizable by anti-A sera and that anti-D serum "170 D" contained neither anaerobiase inhibitor nor anti-collagenase.

DEVELOPMENT OF ε TOXIN IN CULTURES.

Rate of Development of Primary and Potential Toxicity.

When *Cl. welchii* type D is grown in test medium at 37° C., only small amounts of ε toxin appear after 16 to 24 hours; but whereas with poorly toxinogenic strains the ε toxin does not increase thereafter, with highly toxinogenic strains it continues to increase until it reaches a maximum from 3 to 5 days later. Nevertheless, maximal potential toxicity is reach by all strains almost as soon as the growth phase is completed.

These findings are illustrated in Fig. 2, which compares the rate of development of primary and potential toxicity by the toxinogenic (L.A.R.) strain "227" and by the very poorly toxinogenic (H.A.R.) strain "173".

Both strains produced only small amounts of primary toxin overnight ($LD_{100} = 0·25$ ml.); but whereas the L.A.R. strain continued to produce toxin until the primary LD_{100} reached

0·016 ml., the H.A.R. strain remained at the original low level of toxicity. Nevertheless, in cultures of both strains maximal potential toxicity (activated $LD_{100} = 0·002$ ml.) and maximal C.P. were reached overnight.

In subsequent experiments we found that potential toxicity virtually reaches a maximum after about 6 hours but a slight increase may occur up to possibly 30 hours.

Inhibition of Development of ε Toxin in Cultures.

Normal serum. A culture of the L.A.R. strain "W2" grown in test medium to which 25 p.c. of normal ox serum, known to be rich in "antitrypsin", had been added, was compared with a control culture in medium to which 25 p.c. of saline had been added. After incubating for 6 days the primary LD_{100}, activated LD_{100} and A.R. were determined. It was found that the activated LD_{100} of each was 0·001 ml. However, the primary LD_{100} of the control was 0·008 ml., whereas that of the serum medium was 0·125 ml.; hence the presence of serum in the medium caused the A.R. to increase from 8 to 128.

Glucose. It is known that glucose above certain concentrations is unfavourable to production of ε toxin (Dalling and Ross, 1938). In our experiments, strain "W2" was grown in V.F. broth to which glucose ranging from 0·25 to 4·0 p.c. was added. After 6 days' incubation the pH, primary LD_{100} and activated LD_{100} were determined. The results are set out in Table 1.

TABLE 1.

The effect of glucose on production of ε toxin and on A.R.

Glucose (p.c.).	Final pH.	Primary LD_{100}.	Activated LD_{100}.	A.R.
0·25	6·45	0·031	0·002	16
0·5	6·34	0·031	0·002	16
1·0	4·95	$>0·5$	0·008	>64
2·0	4·85	$>0·5$	0·008	>64
4·0	5·30	$>0·5$	0·008	>64

Increasing glucose from 0·25 to 0·5 p.c. caused only a slight fall in pH and did not affect the primary or activated LD_{100}. Increasing glucose to 1 p.c. or more caused a sharp fall in pH and increased the primary LD_{100} to more than 0·5 ml.; at the same time the activated LD_{100} was increased to 0·008 ml. and the A.R. to at least 64. Since proteinase activity of type D decreases rapidly as the pH falls below 7·0, it is likely that the effect of glucose in depressing the primary toxicity and in increasing the A.R. is at least in part due to the fall in pH. It is interesting to note that Taylor and Stewart (1941) found very little ε toxin, even after 6 days, in cultures in their medium containing 1 p.c. of glucose when the pH fell to 5 or less in 24 hours, but if the pH was kept between 7·6 and 7·8 by additions of soda, toxin production was good.

Development of ε Toxin in Cell-Free Supernatants of Young Cultures.

Three toxinogenic strains were sown into test medium. After 6 or 16 hours the cells were removed by strong centrifugation. The primary LD_{100}, activated LD_{100} and A.R. were determined immediately and after anaerobic incubation at 37° C., under toluene, for another 5 days. The following results were obtained.

		Cell-free supernatants examined					
		Immediately.			After 5 days.		
Strain.	Age of Culture.	Primary LD_{100}.	Activated LD_{100}.	A.R.	Primary LD_{100}.	Activated LD_{100}.	A.R.
223	16 hrs.	0·25	0·004	64	0·031	0·004	8
241	6 hrs.	0·5	0·002	256	0·016	0·002	8
326	6 hrs.	0·25	0·002	128	0·031	0·002	16

It is clear that with these strains the continued presence of the cells was not necessary to the development of ε toxin.

With other strains (W2, 206, 211 and 227) we have not succeeded in demonstrating this, and living cells appear to be necessary for the change of prototoxin into toxin. It is possible that with them the living cells play some undetermined rôle in establishing conditions under which extracellular proteinase can act upon prototoxin, both of which reach maximal concentration after a relatively few hours. For instance, young cell-free supernatants from cultures of strain "W2" did not increase in primary toxicity after the cells had been removed, although the pH was practically optimal at 6·8, but when filtrate was incubated with washed cells the primary LD_{100} decreased to one-fourth; on the other hand, cells heated at 60° C. for 30 minutes were without effect.

Correlation between Potential Toxicity and C.P.

Combining power is positively correlated with potential toxicity but has no relationship to primary toxicity, nor to incubation period beyond what is required for the development of maximal potential toxicity. Some observations showing this are set out in Table 2.

TABLE 2.

Relationship between potential toxicity and combining power.

Strain.	Period of incubation (hours).	LD_{100} (ml.) Primary.	LD_{100} (ml.) Activated.	A.R.	Potential toxicity (LD_{100}/ml.).	C.P. (A.U./ml.).
Rose B1	130	0·0415	0·0038	11	263	0·61
170D	24	0·5	0·0024	208	417	1·56
W2	144	0·008*	0·0019	4	526	2·5
170D	22	0·125*	0·0019	66	526	1·92
,,	144	—	0·0015	—	667	2·0
W2	130	0·0060	0·0012	5	833	3·01
170D	144	0·125*	0·0012	104	833	3·01
O'Connor	130	0·0205	0·00095	22	1,053	3·78
W2	24	—	0·0006	—	1,667	7·81

Toxicity and C.P. were determined at 20 p.c. spacings except those marked * which were at 50 p.c. spacings.

The relationship is shown graphically in Fig. 3, which illustrates that all but one of the points lie within the error band ($P = \cdot01$) of the regression line.

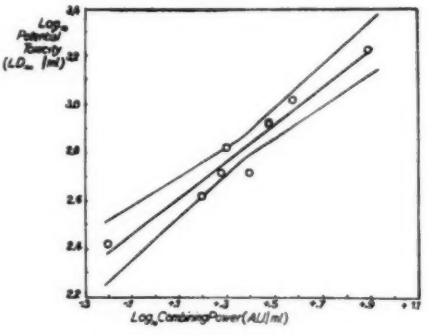

Fig. 3. Relationship between the potential toxicity and the combining power of toxins. Equation of the curve: $y = \bar{y} + b\ (x - \bar{x})$, where $\bar{y} = 2{\cdot}8213$, $\bar{x} = 0{\cdot}3764$ and $b = 0{\cdot}7561$. $P = 0{\cdot}05$ that the true line lies within the limits of the two hyperbolae.

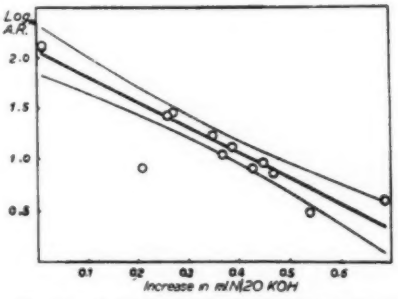

Fig. 4. Relationship between the A.R. and the proteinase activity (with cysteine). Equation of the curve: $y = ae^{bx}$, where $a = 9{\cdot}025$ and $b = -2{\cdot}8571$. $P = 0{\cdot}01$ that the true line lies within the limits of the two hyperbolae.

Correlation Between Proteinase Production and A.R.

In preliminary experiments in which 2 ml. amounts of supernatants were used we found that 5 strains of very high A.R. produced little or no proteinase, whereas 18 strains of medium to low A.R., produced appreciable amounts. However, there was no correlation between the A.R. and the proteinase activity of these 18 strains. It was suspected that this might be due partly to using excessive amounts of enzyme in the digestion mixtures and also that 6 hours' incubation might not be quite sufficient for maximal production of proteinase with all strains. We therefore estimated the proteinase activity of 17-hour culture supernatants of 13 strains of which the A.R. ranged from 3 to 130, using 0·5 ml. of supernatant and cysteine (0·1 p.c.) in the digestion mixtures. Incubation of cultures from which the samples were taken was continued for 6 days after which the primary and activated LD_{100} were determined at 20 p.c. spacings, and the A.R. computed. The results are set out in Table 3.

TABLE 3.

Correlation between proteinase and A.R.

Strain.	Proteinase (ml. N/20 KOH).	Primary LD_{100} (ml.).	Activated LD_{100} (ml.).	A.R.
234	0·69	0·130	0·033	4
326	0·54	0·0048	0·0015	3
230	0·47	0·016	0·0024	7
211	0·45	0·0125	0·0015	9
208	0·43	0·010	0·0012	8
222	0·39	0·0125	0·00095	13
209	0·37	0·008	0·00075	11
237	0·35	0·016	0·00095	17
Rose B1	0·27	0·165	0·006	28
210	0·26	0·026	0·00095	27
227	0·21	0·010	0·0012	8
170D	0·01	0·065	0·0005	130
O'Connor	0·01	0·05	0·00040	125

It is clear that there is no relationship between proteinase activity and either primary or activated toxicity, but that a relationship exists between proteinase activity and A.R. From Fig. 4 in which log A.R. is plotted against increase in ml. N/20 KOH,[1] it is seen that the relationship is exponential, of the form $y = ae^{bx}$, which would be expected if the A.R. is a function of proteinase activity.

It is of interest that the proteinase activity of several strains when examined under standard conditions during periods up to three months was almost constant. This is consistent with our experience that the A.R. usually tends to remain stabilized for considerable periods.

ANTIGENICITY OF ε PROTOTOXIN.

The evidence clearly demonstrates that ε toxin is excreted in cultures as a prototoxin which can combine with antitoxin. A vaccination experiment on lambs was carried out to determine if prototoxin is also antigenic.

Each of 3 groups of 32, 32 and 31 lambs respectively, which had no detectable antitoxin in their sera before vaccination, were injected with one of the alum toxoids *a*, *b* and *c*. Each lamb received subcutaneously a dose of 5 ml. followed after an interval of four weeks by a second dose of 10 ml. The toxoids were prepared by adding 2 p.c. of potassium alum to cell-free culture supernatants which had been detoxicated by incubating at 37° C. with 1 p.c. of formalin for 10 days.

The characteristics of the toxoids used were as follows:

Toxoid.	Strain.	Age of Culture (hours).	Primary LD_{100} (ml.).	Activated LD_{100} (ml.).	A.R.	C.P. of toxoid (A.U./ml.).
a	W2	42	0·031	0·002	16	1·69
b	W2	140	0·008	0·002	4	1·69
c	(i) 170D	140	0·5	0·002	256	1·69
	(ii) 170D	140	0·125	0·001	128	

Toxoids *a* and *b* were prepared from samples of the same culture; toxoid *c* was compounded from two batches so that the C.P. of the mixture was 1·69 A.U./ml.

The A.R. of the respective toxins shows that toxoid *c* was derived almost entirely from ε prototoxin, toxoid *b* mainly from ε toxin, whereas toxoid *a* was derived from prototoxin and toxin in intermediate proportions.

Preliminary experiments had shown that the antitoxic titre reached a maximum about 14 days after the second injection of alum toxoid; therefore serum samples were collected two weeks after the second injection and individually titrated for antitoxin with 20 p.c. spacings in the serum dilutions.

As Smith found with staphylococcal toxoids, the distribution of the antitoxic responses was strongly asymmetrical around the arithmetic mean (A.M.) but the distribution of log (antitoxic response) was approximately normal around the arithmetic mean of the logs, i.e. the log of the geometric mean (G.M.). Logarithms of titres were therefore used instead of the titres them-

[1] Strain 227 which obviously does not conform to the general relationship was excluded from the calculations.

selves in comparing the three toxoids. The titres (A.U./ml.) in group *a* ranged from 0·76–62·5 (G.M. = 5·08); in group *b*, from 0·49–31·25 (G.M. = 2·82); and in group *c*, from 0·19–31·25 (G.M. = 3·93).

Analysis of variance gave the following results:

Source of variance.	Degrees of freedom.	Sum of squares.	Mean square.	F.
Between groups	2	1·069225	0·534612	1·86
Within groups	92	26·451563	0·287517	

The value of F indicated that there were no significant differences between the group means and consequently that there were no significant differences between G.M. of the groups.

The analysis of variance test was made with observations measured on a logarithmic scale and gave a decisive result. It was, however, instructive to supplement this test by classifying the animals in each group according to the magnitude of their antitoxic responses in the original scale, as follows. The A.M. and G.M. refer to the whole 95 animals and were 6·97 and 3·83 A.U./ml. respectively.

Group.	No. in Group.	Toxoid prepared mainly from	Antitoxic response (A.U./ml.). > 1	> 5	> 10	> 20	> A.M.	> G.M.
a	32	ϵ toxin + ϵ prototoxin	31	15	9	3	11	20
b	32	ϵ toxin	28	10	6	1	8	13
c	31	ϵ prototoxin	26	11	9	2	11	20

From the observed distribution of the frequencies in the columns it was obvious that there were no differences between the groups. It may be concluded that there was no evidence that toxoid prepared from cultures poor in toxin but rich in prototoxin (toxoid *c*) was any less efficient than toxoid of the same C.P. prepared from the conventional toxic cultures (toxoid *b*).

DISCUSSION.

In our earlier communication we presented evidence which strongly suggested that most type D toxins contain, in addition to ϵ toxin, an almost atoxic relatively thermostable precursor referred to as prototoxin, which has the same C.P. as toxin and which can be converted into toxin by various extrinsic proteolytic enzymes. We further suggested that in cultures intrinsic proteolytic enzymes might bring about a similar change and promised further evidence in support of this hypothesis. The evidence presented in this communication comprises the following: maximal C.P. and potential toxicity are reached in cultures after only a few hours' growth, whereas primary toxicity may not become maximal until 4 or more days; the addition of normal serum, a known proteinase inhibitor, to the medium depresses the development of primary toxicity without affecting potential toxicity; the addition of 1 p.c. or more of glucose to the medium has a similar effect probably because the pH becomes unfavourable for the activity of type-D proteinases; young cell-free culture supernatants known to contain proteinases, but to be devoid of peptidases, may continue to increase in toxicity when incubated after removal of the cells; combining power is positively correlated with potential toxicity and has no relationship to primary toxicity; an exponential relationship exists between proteinase activity and A.R.; and finally toxoids prepared from toxins of very different primary toxicity but the same potential toxicity and C.P. are equally antigenic.

All the evidence, taken with that already presented in our earlier communication, can be explained by assuming that (a) a precursor of ϵ toxin is excreted from the cells during the growth phase; (b) this precursor is atoxic or relatively so; (c) it is more thermostable than ϵ toxin; (d) it has the same capacity to combine with specific globulin as toxin has; (e) it is equally antigenic; (f) the groups to which it owes its toxicity are "masked" by something that can be digested away by a wide variety of proteinases, including type-D proteinase; and (g) ϵ toxin is derived from ϵ prototoxin and is not excreted directly by the cells.

This process contrasts with the slow release of toxin by *Bact. shigae*, concerning which Okell and Blake (1930) state that "it seems then that toxin production within the cell is entirely a function of the growth phase and that a transfer of the toxin to the filtrate is a secondary autolytic process which runs concurrently with the death and disintegration of the cell".

It might be suggested that ϵ toxin is excreted and is then "masked", but we prefer the simpler hypothesis that the substance excreted is an antigenic but atoxic precursor to which the term ϵ prototoxin may aptly be applied. Whether the prototoxin is converted into toxin in cultures depends upon whether sufficient extracellular proteinase has been excreted and whether suitable conditions for its activity are present. The less these requirements are fulfilled the less prototoxin will be converted. Very few strains excrete sufficient proteinase to convert all the prototoxin and hence nearly all "toxins" can be further activated by trypsin or other suitable proteinases.

This appears to be the first demonstration of a true prototoxin. Cohen, Halbert and Perkins (1942) speculate upon a somewhat similar possibility for the reversibly oxidizable haemolysin of *Str. pneumoniae*. Referring to their observation that during maturation and harvesting, and sometimes during extraction, the haemolytic activity increases many-fold over that found at the end of the logarithmic growth period, they state that "tentatively, we picture the phenomenon as a sort of ripening whereby a reservoir or parent substance is converted, perhaps by cell enzymes, to the haemolytically active material".

We believe that prototoxin is formed not in the medium but in the cells and thence excreted. The recent demonstration that the toxins of *C. diphtheriae* (Pappenheimer, Mueller and Cohen, 1937) and of *Cl. tetani* (Mueller and Miller, 1942) may be produced abundantly in media containing only amino acids, chemically defined growth substances and minerals conclusively proves that at least in those cases toxin is produced not by enzymic breakdown of complex proteoses in the culture medium, as Dernby and Walbum (1923) postulated, but by synthesis; and since ϵ prototoxin appears maximally in the medium before peptidases have been excreted to any significant extent, it is almost certain that the synthesis occurs intracellularly.

The following evidence suggests that the proteinase system of *Cl. welchii* type D consists of two proteinases with the general characteristics of anaerobiase and collagenase:

(a) Normal serum totally inhibited gelatine digestion by many proteinases (Turner and Rodwell, 1943); nevertheless, even when normal serum was increased in the digestion mixtures from 0·6 to 5 p.c., inhibition of proteinase activity of strain "326" could not be increased beyond 27 p.c. Similarly, with two specific antisera it could not be increased beyond 54 and 68 p.c., respectively. Apparently two proteinases were present, one of which is inhibited by a non-specific serum inhibitor and the other by specific antibody.

(b) Normal serum, added to media up to 25 p.c., did not totally prevent the development of ϵ toxin in cultures, although as little as 0·6 p.c. exerted maximal inhibition of gelatine digestion. This suggests the presence of two proteinases, only one of which is inhibited by normal serum.

(c) Proteinase activity of the supernatants of young cultures of 23 strains was increased by cysteine to a variable degree ranging from 11 to 148 p.c. Apparently two proteinases, reacting differently to cysteine are excreted in various proportions. Furthermore the percentage activation by cysteine appears to be characteristic of the strain.

(d) Proteinase activity was not inhibited by iodoacetic acid, but activation by excess cysteine was abolished by iodoacetic acid and by divalent iron. This suggests the presence of two distinct proteinases, one of which may be reversibly activated by cysteine.

Both proteinases must be able to convert prototoxin into toxin (a) because A.R. is correlated with proteinase activity in the presence of cysteine, i.e. collagenase + anaerobiase, and (b) because normal serum does not totally inhibit development of ε toxin in cultures.

The biological function of extracellular proteinases is nutritive: non-diffusible proteins in the environment are broken down to diffusible products. Strains that secrete proteinase are thus less dependent upon symbiosis with better equipped micro-organisms. In the small intestine, where proteins have already been broken down by intestinal enzymes, lack of extracellular proteinase is not important to the nutrition of the micro-organism. It is therefore not surprising that in cases of entero-toxaemia apparently atoxic (H.A.R.) strains may be isolated from highly toxic ileal contents. The discovery of the trypsin activation effect by Bosworth and Glover, and our finding that prototoxin is excreted maximally after only a few hours of growth, offer an acceptable explanation of the possibility of rapid development of enterotoxaemia in animals.

Our results again emphasize the importance of using C.P. as the estimate of antigenicity. Glenny (1931), remarking upon the abandonment of m.l.d. as a measure of the potency of diphtheria toxins and its replacement by C.P. as measured by Lf. states "even now, strangely enough, the strength of tetanus and other toxins is quoted in the literature in terms of m.l.d. without mention of combining power". Strains for use in producing ε toxoid should be selected on their capacity to produce large amounts of prototoxin as measured by C.P. or, more simply, by the toxicity after complete tryptic activation. There is no inherent advantage in using toxinogenic strains for the purpose nor in prolonging incubation after the stage when maximal C.P. or maximal potential toxicity has been reached—at most, 30 hours.

SUMMARY.

ε toxin is not excreted directly by the cells but is derived from a relatively atoxic, antigenic prototoxin which is excreted by the cells during the growth phase, together with extracellular proteinases corresponding to the anaerobiase and collagenase of Maschmann.

On continued incubation ε prototoxin is converted into ε toxin by the proteinases to an extent which varies exponentially as their concentration, if conditions are optimal, and is a fairly constant characteristic of the strain.

The intrinsic proteinases are rarely excreted in sufficient concentration to complete this transformation; hence most "toxins" may be further "activated" by trypsin and certain other proteinases.

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THE METABOLISM OF STARVING LEAVES.

1. PRESENTATION OF DATA; THE NATURE OF RESPIRATION RATE/TIME CURVES IN AIR AND IN NITROGEN AND THE RELATION TO CARBOHYDRATES.
2. CHANGES IN AMOUNTS OF TOTAL AND CHLOROPLAST PROTEINS, CHLOROPHYLL, ASCORBIC ACIDS AND SOLUBLE NITROGEN COMPOUNDS.
3. CHANGES IN MALIC AND CITRIC ACID CONTENTS AND INTER-RELATIONS OF THESE WITH SOLUBLE NITROGEN COMPOUNDS.

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(Accepted for publication 6th February, 1943.)

In earlier papers (Petrie and Wood, 1938a and b; Amos and Wood, 1939; Wood and Petrie, 1938 and 1942; Wood, 1942) a series of experiments was described under conditions such that protein synthesis occurred within leaves. Under the conditions of those experiments changes in amounts of total protein, amino acids and amides were not correlated with changes in the same direction of carbohydrate content; on the contrary increase in amount of protein and of soluble organic nitrogen compounds was associated with decrease in amounts of individual carbohydrates, all of which, but especially sucrose and glucose, may contribute separately to the formation of organic nitrogen compounds. It was inferred that an intermediary compound of carbohydrate degradation, either a glycolytic product or one of the oxidation products of these, and which forms the starting point for synthesis of organic nitrogen compounds, is formed at a rate which may be independent of the carbohydrate concentration.

In all experiments increased external application of ammonium salts resulted in increases in amounts of amino acids and of amides; the latter compounds increase in amount with increase in ammonia content of the leaves until a point is reached when further increase in ammonia content is not associated with further increase in amounts of amino acids or amides: at this point it is probable that the amount of carbohydrate intermediary is limiting.

Total protein content exhibited no discoverable relation with ammonia or carbohydrate contents, but increase in protein content was always associated with increase in amino-acid content and with increase in water content. In all experiments, respiration rate was not correlated with carbohydrate content but showed high positive correlation with amino acid concentration. It was concluded that some amino acids are more readily de-aminated than others bringing about a change in the proportions among the free amino-acids and that this is at least one factor which accounts for the observed relations among protein, amino acids and water contents.

Under the conditions of the experiments described above, carbohydrate content of the leaves was exceptionally high. In the present paper we explore the relations among various metabolites during the hydrolytic phase of protein metabolism brought about by keeping darkened, detached leaves in a gas current at constant temperature. The previous history of the leaves prior to starvation was varied experimentally in some cases in order to induce changes in carbohydrate content. It was our intention to find out how far the conclusions arrived at from consideration of the synthetic phase were valid during the hydrolytic phase of

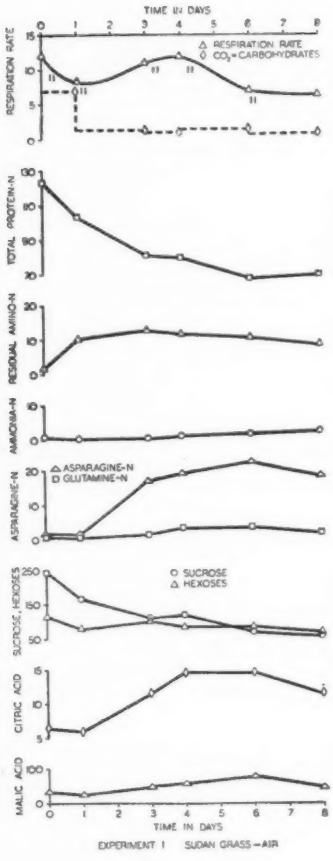


Fig. 1.

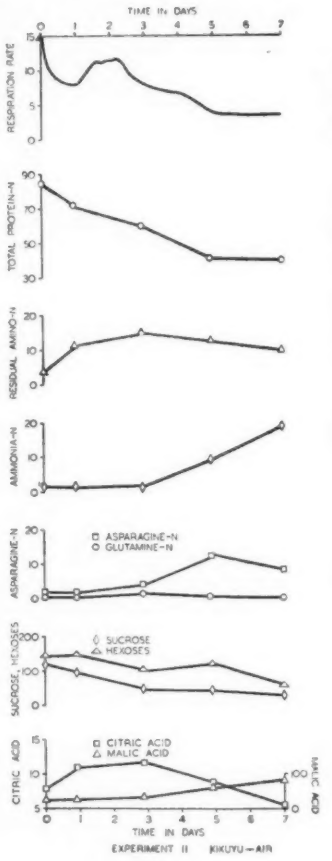


Fig. 2.

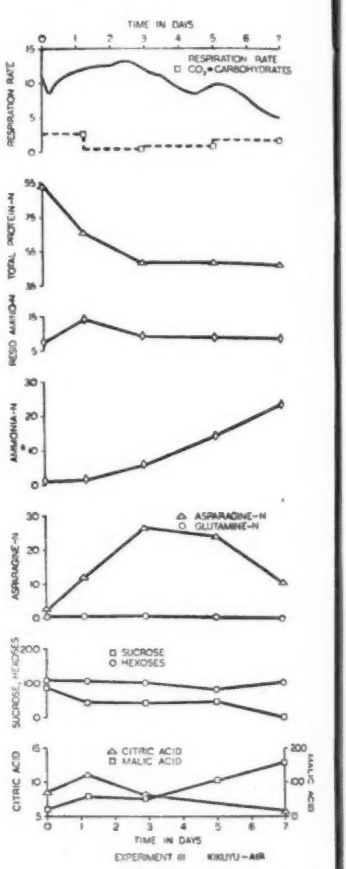


Fig. 3.

protein metabolism; and by extending the range of substances estimated, notably to include the organic acids, to investigate relations between metabolites and to arrive at a scheme indicating the probable course of protein metabolism in leaves. Such a scheme has been proposed in the present paper, so far as is possible from consideration of analytical data only. In the course of our experiments it has been forced upon us that further work on the nature of enzyme systems in leaves and knowledge of the dynamics of reactions concerned are essential for further understanding of the processes.

1. PRESENTATION OF DATA: AND THE NATURE OF RESPIRATION RATE/TIME CURVES IN AIR AND IN NITROGEN.

EXPERIMENTAL.

Method.

The method employed was similar in all experiments. Whole plants were cut either from pots in a glasshouse or from a plot in the field, transferred to the laboratory and the leaves severed at the ligule by a team of workers. The leaves were bulked and thoroughly but carefully mixed;

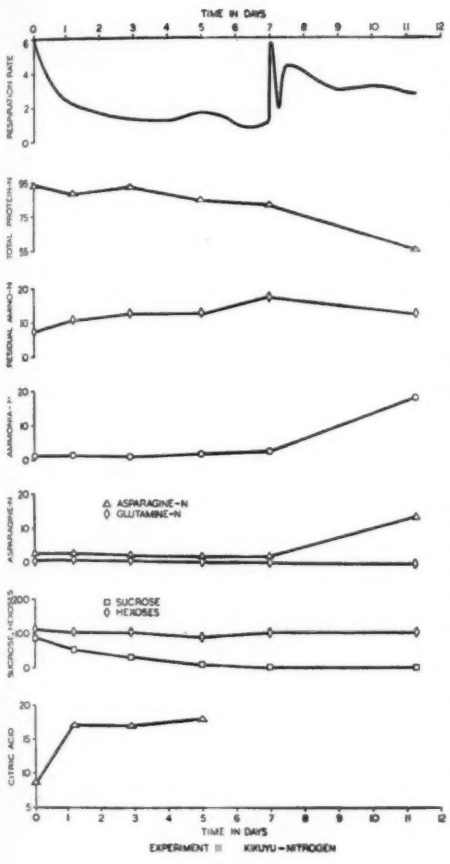
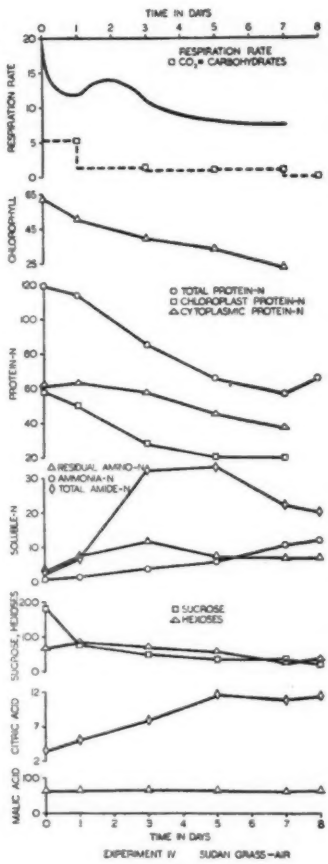


Fig. 4.



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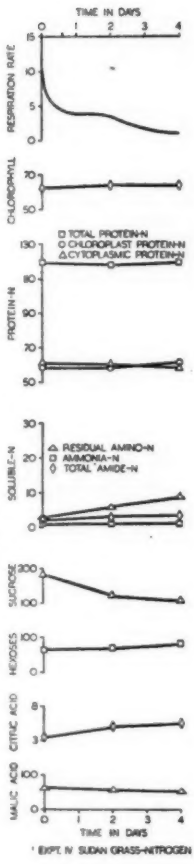


Fig. 6.

samples were weighed and placed in black containers. The latter were placed in water baths at 24·50 ± ·02° C. and a stream of air or nitrogen passed through appropriate flasks for the duration of the experiment. The gas stream was maintained at 4 litres per hour through each flask by means of appropriate needle valves and sensitive manometers. In each experiment leaves were placed in the baths and the gas stream commenced ½ hour after cutting. One pair of samples was used for determination of respiration rate, readings being taken at 2½ hours intervals for the duration of the experiment. At intervals, either of 24 hours or a multiple of this, other flasks were withdrawn from the baths and the leaves subjected to analysis as described at the end of this section. The respiration samples consisted of 20 gm. original fresh weight of the leaves and the analytical samples of 40 gm. original fresh weight of leaves. All results are expressed as mg. per 20 gm. original fresh weight. In some experiments the plants were subjected to preliminary experimental treatments details of which are given in the following section.

Description of Experiments.

The plants used were pure lines of Sudan grass (*Andropogon sudanensis*, L. and B.) and Kikuyu grass (*Pennisetum clandestinum*, Hockst). The former, a summer grass, has been the subject of previous investigations in this laboratory (Barrien and Wood, 1939; Hanson, Barrien and Wood, 1941; Wood, 1941); Kikuyu grass was chosen since we had found that it was able to withstand prolonged exposure in an atmosphere of nitrogen without obvious signs of injury.

Experiment I. Sudan grass grown in pots with nutrients as described by Wood (1941). Experiment commenced 8–4–40 and continued for 8 days. In this experiment continuous records

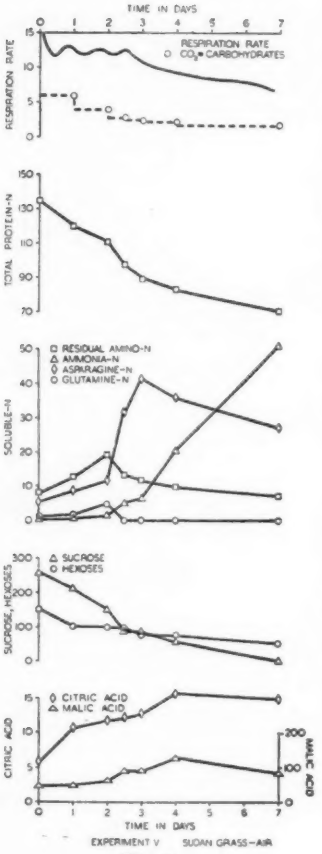


Fig. 7.

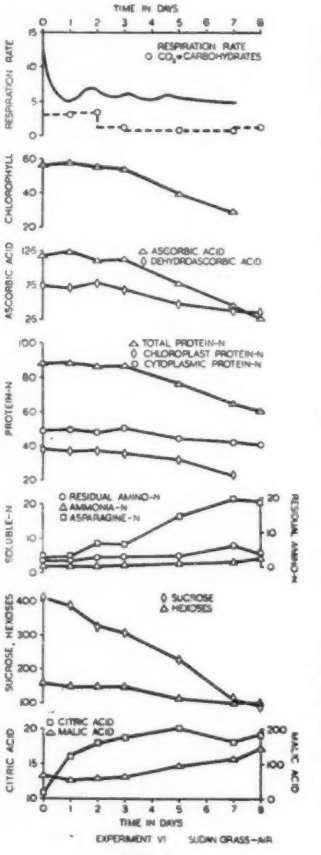


Fig. 8.

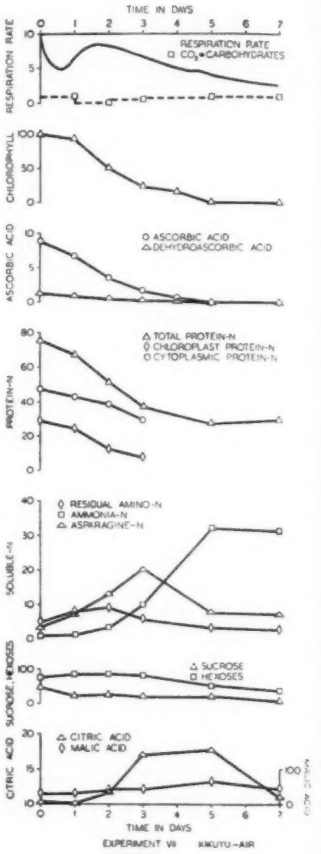


Fig. 9.

of respiration rate were not made, but respiration rate of each sample was determined both in air and in nitrogen prior to removal for analysis. All samples were in nitrogen during the periods indicated. Results of analysis are shown graphically in Fig. 1.

Experiment II. Plants of Kikuyu grass grown in plot in grounds of Botany School. Experiment commenced 2–6–41. Air stream only drawn over both respiration and analytical samples. Results of analysis are shown in Fig. 2.

Experiment III. Kikuyu grass from same plot as Experiment I. Experiment commenced 4–9–41. Both air and nitrogen passed over appropriate samples; air stream continued for 7 consecutive days; nitrogen stream for 7 consecutive days and then replaced by air stream for a further 4 days. After their 7-day sojourn in nitrogen the leaves were fresh, turgid, green and sweet smelling. Results for starvation in air are shown in Fig. 3 and in nitrogen in Fig. 4.

Experiment IV. Sudan grass grown in pots in glasshouse with nutrients as described by Wood (1941). 92 pots were prepared and 5 seedlings per pot planted on 23–2–42. Experiment commenced on 6–4–42 when plants were 47 days old. Four plants were harvested from each pot and formed the material for Experiment IV; the plants had a single stem with an average of 3 leaves per plant. The remaining plants were used later for Experiments V and VI. An air stream was passed over one batch of leaves for 7 days. Over another series nitrogen was passed; these leaves had a brownish tinge on day 2 and on day 4 were approximately half-brown. We had found previously (Hanson, Barrien and Wood, 1941) that Sudan grass was intolerant of exposure to hydrogen. Since injury was suspected the nitrogen stream was stopped on day 4. Results of analysis of leaves in air are given in Fig. 5 and of leaves in nitrogen in Fig. 6.

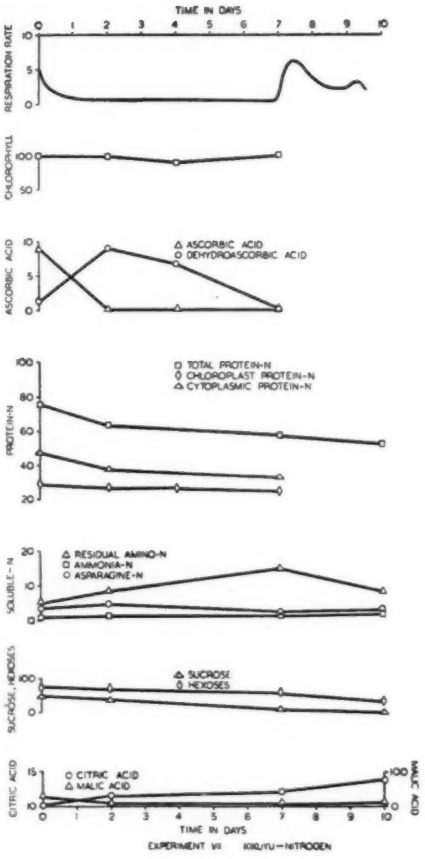


Fig. 10.

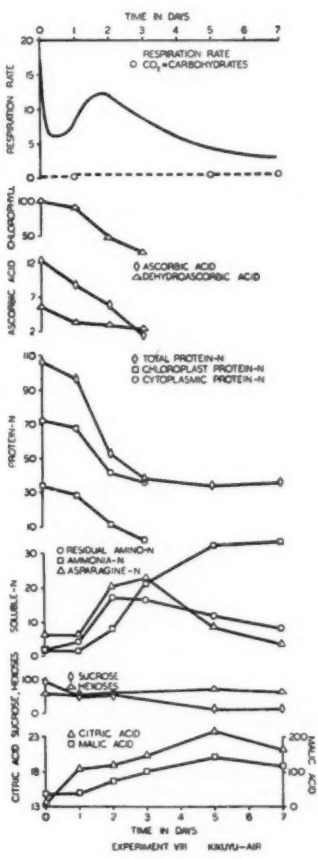


Fig. 11.

Experiment V. Plants of Sudan grass from sowing described in Experiment IV were used: plants were about to tiller and had single stem with 5–6 leaves. On 18–4–42 forty-five pots containing one plant each were placed in constant environment cabinets under constant illumination at a temperature of 24·5° C. for 48 hours. It was hoped in this way to increase the carbohydrate content. On entry into the cabinet the plants contained 146 mg. sucrose and 94 mg. hexoses per 20 gm. fresh weight and after 48 hours contained 259 mg. sucrose and 153 mg. glucose per 20 gm. fresh weight. On 20–4–42 when the plants were 60 days old the leaves were harvested and sojourn in an air stream commenced and continued for 7 days. In this experiment all analyses, with the exception of organic acids, were performed upon the fresh material. Results of analysis are given in Fig. 7.

Experiment VI. Plants of Sudan grass from series described in Experiment IV: plants had exserted inflorescences 20 days previously and leaves were long, strap-like and dark-green. The plants were placed in the constant environment cabinet on 16–5–42 for 48 hours; at the end of this period sucrose had reached the high value of 409 mg. per 20 gm. fresh weight. The leaves were harvested on 18–5–42, when the plants were 90 days old, and an air stream passed over the samples for 8 days. Results of analysis are given in Fig. 8.

Experiment VII. Plants of Kikuyu grass were grown in a plot in grounds of Botany School Experiment commenced 15–6–42. Both air and nitrogen passed over appropriate samples: air stream continued for 7 consecutive days; nitrogen stream for 7 consecutive days and then replaced by an air stream for a further 3 days. At the end of their seven-day sojourn in nitrogen

the leaves were green with slight brown colouration and had a slight garlic-like odour somewhat reminiscent of that of methionine; this smell disappeared after the subsequent exposure of the leaves to the air stream. Results for starvation in air are given in Fig. 9 and in nitrogen in Fig. 10.

Experiment VIII. Plants of Kikuyu grass from same plot as in Experiment VII. Experiment commenced 31–8–42. An air stream was passed over appropriate samples: air stream continued for 7 consecutive days. Results for starvation in air are given in Fig. 11.

Analytical Methods.

Respiration rate. 20 gm. fresh leaves were placed in black containers in a water-bath at 24·50 ± ·02° C. Air was obtained from outside the laboratory by means of a pipe-line and blown, only slightly above atmospheric pressure, over the leaves and through the absorbing liquid. Nitrogen from cylinders was passed over copper spirals in an electrically-heated quartz furnace. All gas streams were freed from CO_2 by passage through soda-lime tubes, passed through water-bubblers and cooled to bath temperature before entrance to the leaf chambers. Respiration rate was determined by measuring amount of CO_2 produced over definite time intervals. In Experiments I–III CO_2 evolved was measured at frequent intervals by the electrical conductivity method described by Wood (1941): in Experiments IV–VIII, CO_2 was absorbed in N/10 NaOH in Pettenkofer tubes and the contents subsequently titrated with N/10 HCl in presence of excess $BaCl_2$. Every 2½ hours the gas streams were diverted to fresh tubes by means of a modification of the gas-commutator described by Blackman and Parija (1928).

Chloroplast protein-N. Pure samples of chloroplasts were isolated from 20 gm. samples of fresh leaves by the centrifugation method described by Hanson (1941) and Hanson, Barrien and Wood (1941). After extraction of the pigments, protein-N was determined as described by Hanson, Barrien and Wood (1941).

Total protein-N was determined on leaf material, rapidly dried in an air-stream at 90° C., by the method described by Hanson, Barrien and Wood (1941). In Experiment V, total protein was determined on fresh material by the method described by Petrie and Wood (1938). Cytoplasmic protein is recorded as the difference between total and chloroplast-proteins.

Soluble-nitrogen fractions. Ammonia-N, glutamine-N, asparagine N and total amino-N were determined in aliquots of the protein-free filtrate by methods outlined by Wood (1941). Glutamine-N and asparagine-N are recorded as twice the value of the respective amide-N. Residual amino-N was obtained by deducting the amide-N of the amides from total amino-N.

Chlorophyll was determined in the isolated pure chloroplasts and also in aliquot samples of the fresh material by the method described by Hanson (1941). In Experiments VII and VIII relative values only are given, the amount of chlorophyll in the initial sample on day 0 being taken as equal to 100 p.c.; in other experiments values are given in absolute units.

Carbohydrates. Reducing sugars before and after hydrolysis with acid were determined in the protein-free filtrate by the method of Hagedorn and Jensen as outlined by Amos and Wood (1939). The amount of reduction before hydrolysis, calculated as glucose, we have labelled "hexose". The increase in reduction after hydrolysis, calculated as glucose, we have labelled "sucrose" although it also includes the fructosan fraction. In Experiment V fresh leaves were plunged into boiling alcohol and the extracts consequently contained no fructosan.

Ascorbic and dehydroascorbic acids were estimated in 5 gm. samples of the fresh material by grinding leaves with metaphosphoric acid, reduction of some aliquots with H_2S, and subsequent titrations with 2: 6 dichlorphenol-indo-phenol in the presence of formaldehyde according to the procedures described by Lugg (1942).

Citric acid. Organic acids were extracted according to the method of Pucher, Vickery and Wakeman (1934a). Citric acid was determined by the method of Pucher, Vickery and Wakeman (1934b).

Malic acid. In Experiments I–III l-malic acid was determined by the polarimetric method of Vickery and Pucher (1933) in the presence of uranium acetate. In all other experiments malic acid was determined by the 2: 4 dinitrophenylhydrazine method of Pucher, Vickery and Wakeman (1934b). Colorimetric comparisons were carried out in a Coleman spectrophotometer[1] at a wave length of 580mμ.

Note on Metabolism in Nitrogen.

In this paper we have been concerned primarily with inter-relations among metabolites when leaves are starved in air. Experiments with starving leaves in nitrogen were designed originally to reproduce with leaves the old experiment of Godlewski (1904) with seedlings and demonstrate directly the secondary origin of amides from amino-acids. The data in nitrogen presented here make no pretensions to be detailed studies and it is clear that more frequent analyses are desirable during the sojourn in nitrogen and during the aftermath in air; the changes in the

[1] We are indebted to Mr. H. R. Marston, Director of the Animal Nutrition Laboratory, C.S.I.R., University of Adelaide, for the use of the Coleman spectrophotometer.

ascorbic acids also require further investigation. We have included our somewhat inadequate data here because they throw light upon the course of respiration rate, nitrogen metabolism and organic acid metabolism.

THE NATURE OF RESPIRATION RATE/TIME CURVES.

The form of the respiration rate/time curves in air shows similar characteristics in all experiments and is analogous to those described by Yemm (1935) for barley and by Godwin and Bishop (1927) for cherry-laurel leaves. The curves show three well-marked phases: (1) an initial rapid decrease in rate, (2) a climacteric during which there is an increase in rate or maintenance over a period of an approximately constant rate and (3) a senescent decrease in rate.

Differences in the nature of the climacteric are evident in the experiments. In all experiments with Kikuyu grass (Figs. 2, 3, 8, 9) the climacteric is characterized by a well defined "hump" similar to that described by Godwin and Bishop (1927) for cherry-laurel; in Sudan grass "humps" occur in some cases (Figs 1, 5) but in others (Figs. 7, 8) respiration rate remains practically constant throughout the climacteric period. The incidence and duration of the climacteric varies in the different experiments. Possible reasons for these differences as well as for the form of the climacteric, are deferred for consideration below.

In nitrogen the form of the respiration rate/time curve is entirely different. Respiration rate decreases progressively with time and the form is approximately logarithmic. During early stages of starvation there occurs a rapid decrease in rate and this is followed by a slower decrease. No climacteric rise, as is characteristic in air, takes place. On replacing the nitrogen passing over starving leaves with air, there is a rapid increase in rate of CO_2-production to a high level, many times that of the previous rate in nitrogen; this is followed by a decrease in respiration rate. This phenomenon in air appears to be a delayed climacteric rise.

RELATION OF RESPIRATION RATE TO CARBOHYDRATES.

Soluble carbohydrates only have been determined in these experiments and individual carbohydrates were not determined separately; "hexoses" include glucose and fructose and "sucrose" includes both sucrose and soluble fructosans. In common with other grasses it is probable that some starch and considerable amounts of hemicelluloses, which may enter into the metabolic flux, were present.

In general the curves for sucrose/time in air exhibit two well marked phases: (1) an initial, relatively rapid and approximately linear decrease with time, and (2) a phase during which sucrose decreases only slowly with time. In some cases (e.g. Figs. 1, 4, 5, 9) the latter phase appears to be grouped into two stages; an earlier one following the initial fall and a later one during which sucrose disappears more rapidly. Hexoses remain approximately constant in amount during early stages of starvation but later decrease in amount. The duration of the early rapid fall in sucrose depends on the initial content (cf. Experiments V and VI, when sucrose content is initially high, with other experiments where it is much lower).

In nitrogen decrease in amount of sucrose with time is approximately logarithmic, but hexoses remain approximately constant in amount throughout the starvation time. The rate of disappearance of carbohydrates is more rapid in nitrogen than in air.

It is uncertain whether the initial high respiration rates in air represent the true respiration rates of leaves at time of cutting of the samples; for Audus (1935), Godwin (1935) and Barker (1935) have all shown that handling leaves caused a marked rise in respiration rate followed by a rapid fall. Experiment VI seems to indicate that the initial decrease in rate may be of this kind. In this experiment, after the initial rapid fall the respiration rate shows only a small decrease over the whole experimental period; during the first 72 hours of this time there was little significant change in soluble nitrogen compounds, such as occurs in other experiments, and which could not therefore act as substrates in respiration: carbohydrates were the only substances estimated which showed significant decrease in amount over this period. It is possible, therefore, that the approximately constant respiration rate over the first 72 hours may be determined by carbohydrate content only and represents the rate at time of cutting the samples. Wood and Petrie (1942) have advanced evidence to show that when carbohydrate content is high then respiration rate is independent of carbohydrate content.

Whatever the cause of the initial decrease in respiration rate it is clear that it accompanies the initial rapid fall in sucrose but is not co-incident with the latter. Reference to the figures shows that the climacteric rise commences before the initial rapid fall in sucrose is completed; this is most marked in Experiments V and VI (Figs. 7, 8) where initial content of sucrose is high. This fact seems to indicate that substrates other than carbohydrates estimated undergo oxidation during the climacteric. The period of relatively small decrease in sucrose content is also included within the period of the climacteric rise, except in Experiment VI where carbohydrate content was high; this also suggests utilization of other substrates and a "sparing" of carbohydrates during the climacteric.

From the values for soluble carbohydrates at consecutive time intervals in each experiment and assuming that the carbohydrates are oxidized to CO_2 and H_2O, the amount of CO_2 corres-

ponding to carbohydrate loss can be calculated. This CO_2-equivalent, expressed as a mean hourly rate is given in the figures for each experiment. In no instance does the CO_2-equivalent of carbohydrates estimated equal the CO_2 evolved in respiration. In air a greater proportion of the CO_2 evolved is accounted for in terms of sucrose during the initial stage than during the climacteric when carbohydrates apparently contribute an almost negligible fraction of the CO_2 evolved. These results are in agreement with those of Yemm (1935) who found that only during the first few hours of starvation could rate of CO_2-production in barley leaves be accounted for in terms of total carbohydrates; Yemm also found in barley leaves that the ratio CO_2/O_2 suggested oxidation of carbohydrates only during the initial stage of starvation. Deleano (1912) also found that more CO_2 was respired than could be accounted for by complete oxidation of all carbohydrates and organic acids in leaves; and Vickery *et al.* (1933), although they did not measure respiration rate, concluded that soluble carbohydrates played a minor rôle in CO_2-production during yellowing of tobacco leaves.

Yemm (1937) put forward the view that during later stages of starvation soluble nitrogen compounds act as substrates in respiration and this suggestion is supported by our data. In each experiment the duration of the climacteric rise extends from the cessation of residual amino-acid increase to cessation of asparagine increase, i.e. it corresponds to the period during which amino acids derived from protein hydrolysis are oxidized and the ammonia formed built into asparagine. The decrease in respiration after the climacteric is accompanied by decrease in amide and residual amino acids as well as in carbohydrate content.

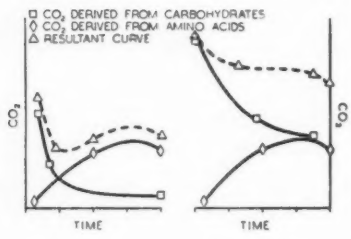


Fig. 12a. **Fig. 12b.**

The form of the climacteric might be expected to depend therefore on the relative amounts of CO_2 contributed by carbohydrates (which are decreasing in amount) on the one hand and by soluble nitrogen compounds (which are increasing in amount) on the other. In our experiments a climacteric "hump" is always associated with a low and falling sucrose content and a "constant" climacteric is associated with relatively high and falling sucrose content. The two possibilities are illustrated in Fig. 12a and b. In each case the CO_2 contributed by amino acids is assumed to be the same; in Fig. 12a the carbohydrate content is low and rapidly decreases whilst in Fig. 12b carbohydrate content is high. In the former case the resultant curve is "humped", but prolonged in the latter case.

Another fact evident in the data should be pointed out here. In the batch of Sudan grass plants, respiration rate is much lower at all periods in the old leaves of Experiment VI than in the young leaves of Experiments IV and V, although the carbohydrate content is much higher in the old leaves. It has been suggested (Richards, 1938; Petrie and Williams, 1938; Wood, 1941) that the decrease in respiration rate with increasing age may be due to associated decrease in protein content which might be a measure of the number of respiratory centres. It should be pointed out however that Marsh and Goddard (1939) have reported a quantitative change in oxidizing enzyme systems with increased age of leaves.

CONCLUSIONS.

The conclusions drawn from the work described in this part are as follows: In nitrogen and during early stages of starvation in air, change in respiration rate is associated with change in amounts of sucrose (or probably more accurately in amounts of furanoses). The climacteric rise in respiration rate occurs only in air and is associated with relatively small utilization of carbohydrates as respiratory substrate, but is associated with oxidation of amino acids and formation of asparagine. The time of onset of the climacteric rise is determined by the initial sucrose content and its form by the relative amounts of sucrose and of amino acids present.

2. CHANGES IN AMOUNTS OF TOTAL- AND CHLOROPLAST-PROTEINS, CHLOROPHYLL, ASCORBIC ACIDS AND SOLUBLE NITROGEN COMPOUNDS.

TOTAL PROTEIN.

In all experiments in air with the exception of Experiment VI, and as in the experiments of Vickery *et al.* (1933) and of Yemm (1937), protein loss commenced immediately the leaves were placed in darkness. But in Experiment VI (Fig. 8) loss of protein did not assume significant

amount until the leaves had been at least three days in darkness. Experiment VI differed from other experiments with Sudan grass in that preliminary sojourn in the cabinet resulted in accumulation of a large amount of sucrose. This experiment supports the observations of Deleano (1912) and Meyer (1918) who, working with detached leaves, found that proteolysis began only after the carbohydrate supply had become relatively low. Experiment VI is also in accord with the observations of Wood and Petrie (1942) on plants grown under conditions such that synthesis was proceeding, that when the carbohydrate content is very high, increase in protein content is associated with decrease in amounts of sucrose and other carbohydrates.

Explanation of the so called "protein-sparing effect" when carbohydrate content is high may be that carbohydrate intermediaries which form the starting point for amino acid synthesis are formed and converted into amino acids at a rate greater than that of oxidation of amino acids.

Smirnow and Izwoschikow (1930) found that the total protein content of young tobacco leaves decreased more rapidly during starvation than that of older leaves and concluded that during starvation a relatively labile reserve-protein was broken down whilst a more stable plasma-protein was more resistant to hydrolysis. Vickery, Pucher, Wakeman and Leavenworth (1933) found that protein digestion was rapid during early stages of curing tobacco leaves but diminished when leaves entered the yellow stage and could no longer be detected when leaves became brown. They also found that the ratio of amino-N after complete hydrolysis to total-N of the protein diminished during early stages of curing but remained constant when the yellow stage was reached. Vickery *et al.* therefore supported the view of Smirnow and Izwoschikow that a relatively labile protein was utilized during early stages of starvation. Yemm (1937), studying starving barley leaves found that completion of yellowing corresponded with a marked reduction in rate of protein hydrolysis and suggested that this might be caused by exhaustion of proteins associated with the chloroplast.

In the experiments of Smirnow and Izwoschikow 35 p.c. of the total protein-N was hydrolyzed after 5 days, when their experiments ended; Vickery *et al.* found that 45–50 p.c. of the total protein had disappeared on entry of leaves into the yellow phase; Yemm's figures indicate that 50–60 p.c. of the total protein was hydrolyzed at the end of the yellow phase of starvation.

In the experiments described in this paper the course of protein loss in air is similar to that described by Vickery *et al.* and by Yemm. With the exception of Experiment VI, loss of protein commences during early stages of starvation, the rate of loss being approximately constant until onset of yellowing when the rate of loss progressively decreases. At the end of the yellow phase 40–60 p.c. of the total protein has been hydrolyzed.

In an attempt to study the utilization of different proteins during starvation we have isolated and determined the amount of chloroplast-protein in addition to estimating the total protein.

CHLOROPLAST-PROTEIN AND OTHER CONSTITUENTS OF THE CHLOROPLAST.

Chloroplast-protein has been determined in Experiments IV, VI, VII and VIII (Figs. 5, 8, 9, 11). In air chloroplast-protein began to decrease in amount as soon as the leaves were placed in darkness, except in Experiment VI where, owing to high carbohydrate content, decrease in amount commenced only after 3 days.

Examination of the data shows clearly that the plants may be divided into two classes according to the behaviour of the chloroplast protein: (1) in Sudan grass (Figs. 5, 8) chloroplast protein decreases more rapidly in amount than does cytoplasmic protein; (2) in Kikuyu grass (Figs. 9, 11) both chloroplast and cytoplasmic proteins decrease in amount at approximately the same rate.

In air other attributes of the chloroplasts decrease in amount concurrently with decrease in amount of chloroplast protein (Figs. 5, 8, 9, 11). Chlorophyll content and ascorbic acid content both show closely similar drifts and decrease in amount as the chloroplast protein decreases. Dehydroascorbic acid also decreases in amount with increasing time of starvation, but less rapidly than does ascorbic acid. It should be noted in Experiment VI that chlorophyll and ascorbic acid do not commence to decrease in amount until day 3 when decrease in amount of protein also commences.

Recent work (Weier, 1938) has focussed attention on the idea of the existence of a pattern within the chloroplast in which protein, chlorophyll, carotinoids, ascorbic acid and phosphatides are all inter-locked components. Our data support the idea of such a pattern since all components decrease at approximately equal rates during starvation.

During isolation of the pure chloroplasts we had noticed that with the onset of yellowing many of the chloroplasts disintegrated during extraction and centrifugation. We therefore followed changes in chloroplasts during the course of starvation by examining sections of leaves under a microscope. During early stages of starvation prior to yellowing and whilst protein, chlorophyll and ascorbic acid are decreasing in amount the chloroplasts shrink considerably; with the onset of yellowing the chloroplasts begin to disintegrate and this disintegration is practically complete when full yellow colour has been reached. The chloroplast fragments, coloured yellow with carotinoid oxidation products, may be seen lying in the cytoplasm during early stages of the yellow phase. The possibility therefore that enzymes are destroyed or liberated into the cytoplasm during disintegration must be borne in mind.

BEHAVIOUR OF PROTEINS IN NITROGEN.

Before discussing possible reasons for the breakdown of chloroplast components in air it is necessary to study their changes when leaves are starved in an atmosphere of nitrogen. In Experiments III and VII (Figs. 4, 10) leaves of Kikuyu were placed in a stream of nitrogen for periods up to seven days when the nitrogen was replaced by air. In each case, on exposure to air the respiration rate rose to a high value and over the subsequent days exhibited features usually associated with the climacteric. At the end of the sojourn in nitrogen leaves in Experiment III were fresh and turgid and contained whole chloroplasts, the same features were evident in Experiment VII although the leaves were slightly brown. On exposure to the air stream amides and ammonia were formed in the manner characteristic of leaves placed initially in air. During the period in nitrogen the sucrose content decreased progressively and at a somewhat faster rate than in air; in Experiment III the amount of sucrose fell to zero at the end of the period in nitrogen. For these reasons there is no evidence that the leaves were injured during their sojourn in nitrogen.

In Sudan grass on the other hand, the leaves rapidly became quite brown on exposure to nitrogen and the intercellular spaces appeared to be injected. It had been found previously (Hanson, Barrien and Wood, 1941) in attempting to measure rate of carbon assimilation in Sudan grass leaves in hydrogen that the rate fell off rapidly with time and the leaves became brown. During early stages of exposure to nitrogen the course of metabolism in Sudan grass leaves (Fig. 6) is apparently similar to that in Kikuyu; but on account of the obvious signs of injury to Sudan grass we have restricted our observations in nitrogen to Kikuyu.

In Experiment VII ascorbic and dehydroascorbic acids decrease in amount with increased time of starvation.

In the young leaves of Experiment VII the ascorbic acid was rapidly and completely converted into dehydroascorbic acid which then progressively decreased in amount. How far this conversion is characteristic or whether it is restricted to young leaves cannot be determined on the evidence available. Such a conversion is not surprising since the ascorbic system is the most strongly reducing one present in the cell and may accept oxygen in a number of reactions. A possible explanation of the browning observed in Experiment VII is the well known oxidation of quinones to brown-coloured products by the ascorbic acid-ascorbic oxidase system.

Chlorophyll content shows no appreciable change during sojourn of leaves in nitrogen.

Protein progressively decreases during sojourn in nitrogen, but the loss is extremely small compared with losses over the same period in air (cf. Experiment III, Figs. 3, 4; Experiment VII, Figs. 9, 10). When the nitrogen is replaced by air more rapid breakdown of protein commences. The changes in amount of protein are so small during the periods studied that it cannot be said whether chloroplast or cytoplasmic proteins or both are concerned. It will be shown in the following section that residual amino-acid-N appears in the leaves in amount approximately equivalent to the amount of protein lost, except in Experiment VII where initially protein loss is greater than soluble nitrogen compounds estimated and may be due to peptide formation (cf. Vickery *et al.* with tobacco leaves in air). Amides and ammonia do *not* increase in amount as is the case during starvation in air.

It is apparent therefore that the primary cause of decrease in amount of protein in air is oxidation of amino acids derived from the protein. The view that protein content is determined by carbohydrate content or by energy derived from respiration cannot be maintained in view of the data for Experiment VI and for leaves in nitrogen. In air sucrose content of the leaves may be a factor determining the onset of protein hydrolysis for as discussed above when sucrose content is high it may supply the non-nitrogenous skeleton of amino-acids at a rate greater than the rate of oxidation of amino acids.

It is also apparent that the chloroplast is the seat of many oxidations; it is probable that proteases and amino-oxidases are present in the plastid, but whether other systems associated with the various oxidations are present or even localized in the plastids cannot be said at present.

The small amount of protein breakdown which occurs in nitrogen raises the question of the relation of proteases to proteins.

The formation of the peptide linkage of proteins requires absorption of energy which has usually been considered to be derived from respiration. It might be expected therefore that protein hydrolysis should be marked in nitrogen. Possible explanations are in terms of spatial separation of enzymes and substrate or of "steric hindrance" whereby the protein in large measure is protected from hydrolysis by enzymes on account of the pattern which it forms with phosphatides, chlorophyll, carotinoids and ascorbic acid. This explanation receives support from the observations of Strain (1938) that xanthophylls and chlorophyll are not readily oxidized in the living leaf but undergo rapid oxidation when the leaves are ground with sand and the chloroplast structure destroyed.

AMINO ACIDS AND AMIDES.

The amount of residual amino-acid is measured by total soluble amino-N *minus* the amino-N of the amides. In this discussion the amount of amide hydrolyzed at pH 6·5 is referred to as "glutamine"; the more stable amide hydrolyzed at pH 1·0 is referred to as asparagine.

There is a similarity between the nitrogenous metabolism of starving leaves in air and that described by Schulze (1878) and Prianischnikow (1899) for seedlings grown in darkness. A series of events occurs during starvation in air. The work of Vickery *et al.* (1933), Mothes (1926) and Yemm (1937) with leaves of tobacco, bean and barley respectively agrees in showing that during early stages of starvation, and when carbohydrates are relatively low, there is a decrease in protein-N and a simultaneous increase in amino-N; later the amides asparagine and glutamine make their appearance and later still ammonia accumulates within the leaves.

Our experiments conform to this picture in that maximum residual amino-acid content is attained before the maximum content of glutamine and asparagine and these in turn before the maximum ammonia content. But there is no evidence of sudden appearance of new reactions, which indeed are difficult to conceive, except in the case of possible sudden liberation of enzyme systems such as might accompany disintegration of the chloroplasts but the series of changes outlined above generally precedes chloroplast disintegration. On the contrary increase in the content of amides and of ammonia accompanies protein degradation from the start. Amide accumulation in general commences at a relatively slow rate, this is followed by an increased rate of formation until a relatively steady rate is reached and maximum amide content is attained, after which the amount of amide decreases with time. Glutamine is present in the leaves in such small amount that its changes cannot be followed with great accuracy; as noticed by Yemm its maximum is often earlier than the asparagine maximum. The attainment of maximum asparagine content in all cases is contemporaneous with disintegration of the chloroplast and with the slowing down in rate of protein breakdown (*vide* Table 1). Ammonia accumulates relatively slowly during the stages of residual amino acid and of amide formation but accumulates rapidly during the stage of loss of asparagine.

In light in the presence of abundant carbohydrates Mothes (1926) showed that added ammonium salt was used in amide formation. Petrie and Wood (1942) showed that under these conditions residual amino acids, glutamine and asparagine are formed and that the amounts of these substances exhibit high positive correlations with one another. It has generally been assumed (Vickery *et al.*, 1933; Yemm, 1937) that in air asparagine and glutamine are derived from sugar and from amino acids by oxidation of the latter to ammonia and subsequent conversion of this combined with carbohydrate degradation products, probably through aspartic and glutamic acids, to the amides; though both authors discuss the possible liberation of amides preformed in the protein molecule.

It seemed to us that these points could be more readily substantiated by repeating the experiment of Godlewski (1904), but using leaves instead of seedlings in an atmosphere of nitrogen. Under these conditions, and under circumstances wherein the leaf tissues are not injured, the amount of protein loss is continuous but small and amino acids only accumulate; amides do not accumulate but on the contrary decrease slightly in amount, nor does accumulation of ammonia occur. It is clear therefore that the amides do not arise preformed from protein but by oxidation of the amino acids.

It has been pointed out previously that the climacteric hump in respiration occurs between maximum contents of residual amino acid and of asparagine and that the onset of the climacteric is determined by the initial carbohydrate content. It is apparent that the onset of asparagine accumulation and the maximum asparagine content are also correlated with the initial sugar content (*vide* Table 1), and the higher the sucrose content the later the asparagine maximum. It has been pointed out above that one explanation of this might be that when sucrose is high one or more amino acids are formed at a rate greater than their oxidation to ammonia. It is proposed to consider first further evidence for this view and to postpone discussion of the possible mechanism of amide formation until the data for the organic acids have been presented.

POSSIBLE MECHANISM OF PROTEIN DEGRADATION.

In seeking an explanation of the mechanism of protein breakdown and of the difference in behaviour during breakdown of the cytoplasmic proteins of Sudan grass and Kikuyu grass, it is pertinent to examine the metabolism of sulphur during starvation. Wood and Barrien (1939b) showed in rye grasses during starvation in air that the amount of protein-S progressively decreased with time of starvation, loss of protein-S occurring concurrently with loss of protein-N. At the same time an equivalent amount of inorganic sulphate-S appeared in the leaves whilst the amounts of soluble organic-S compounds (cystine, methionine and glutathione) remained approximately constant. Wood and Barrien (1939a) have also shown that when cystine is added to the soil in which grass plants are growing then no increase in amount of protein-S or of cystine occurs within the leaves but the content of inorganic sulphate-S in the leaves increases in amount. Petrie and Wood (1938a) have shown that when the water content of plants is reduced, protein hydrolysis takes place and the amino-acid content increases, but this increase in total amino acid content is not accompanied by increase in amount of cystine.

It is possible therefore that the proteolysis observed during starvation and during wilting might be brought about by oxidation of cystine at a rate greater than its formation. When sucrose concentration is high, as in Experiment VI, the non-nitrogenous precursors may be produced and condensed with ammonia and sulphydryl compounds at a rate equal to or greater than cystine

oxidation, or alternatively a relatively high respiration rate resulting from high carbohydrate content may provide energy for sulphate reduction at a rate greater than oxidation of cystine. Oxidation of cystine would result in a disproportionality among free amino acids causing protein to hydrolyze to restore equilibrium (cf. Petrie and Wood, 1938b).

This hypothesis also provides an explanation for the different behaviours of the leaf proteins of Sudan grass and by Kikuyu grass. Hanson, Barrien and Wood (1941) have shown that in Sudan grass the chloroplast-protein is rich in sulphur, containing approximately 70 p.c. of the total protein-S. If cystine produced by hydrolysis is preferentially oxidized it would be expected that chloroplast protein would be more rapidly broken down than the sulphur-poor cytoplasmic protein. On the other hand in *Lolium subulatum*, Wood and Barrien (1939b) have shown that there is no significant change in the ratio Total Protein-N/Total Protein-S during starvation which would seem to indicate that cytoplasmic- and chloroplast-proteins have similar protein-S contents. It is possible that Kikuyu is similar to *Lolium subulatum*. In the case of Kikuyu however this hypothesis does not explain completely the cessation of protein hydrolysis at the time of disintegration of the chloroplasts. It is possible therefore that a portion of the enzyme systems, e.g. proteolytic enzymes, is destroyed with the change in organization of the chloroplasts.

CONCLUSIONS.

The evidence presented in this section shows that, except where carbohydrate content is high, the total protein content of starved leaves decreases regularly with time to a value from which there is only slight subsequent fall with time. The latter period approximates to the stage of full yellowing of the leaf. In leaves rich in carbohydrates decrease in amount of protein does not commence until the carbohydrate content has decreased to a relatively low amount.

In Sudan grass chloroplast-protein decreases in amount more rapidly than cytoplasmic proteins; but in Kikuyu grass both cytoplasmic- and chloroplast-proteins decrease at approximately the same rate. Chlorophyll content and ascorbic acid contents decrease in amount contemporaneously with decrease in chloroplast-protein content and cessation of protein hydrolysis is coincident with chloroplast disintegration. The chloroplast protein of Sudan grass contains most of the total protein-S whilst in Kikuyu it is suggested that both chloroplast- and cytoplasmic-proteins have approximately the same sulphur content.

It is suggested that at least one factor causing protein degradation is oxidation of the amino acid cystine to sulphate at a rate greater than that of its formation.

In nitrogen protein content decreases only slowly with time and an equivalent amount of amino acids is produced but no amides are formed; in air the amount of protein decreases rapidly and the amino acids first formed are oxidized to glutamine and asparagine. Later the amides decrease in amount and there is a marked accumulation of ammonia.

3. CHANGES IN MALIC AND CITRIC ACID CONTENTS AND THEIR INTER-RELATIONS WITH SOLUBLE NITROGEN COMPOUNDS.

Relations between malic and citric acids and of these with other metabolites are complex. In Table 1 we have collected data from the experiments showing times after commencement of starvation at which malic and citric acids, as well as other metabolites reach their maximum contents. Examination of this Table and of the individual experiments makes it clear that there is no linear correlation between amounts of malic and citric acids nor are the times of maximum content of the two acids coincident. In general, the maximum content of citric acid occurs earlier than the maximum malic acid content.

CITRIC ACID.

The production of citric and malic acids in the leaf is associated with reactions taking place in the presence of oxygen. In all experiments there is little change in amount of citric or malic acid during the sojourn in nitrogen. A small increase in amount of citric acid associated with a small decrease in malic acid occurs during the early stages of starvation in nitrogen and this may be associated with decrease in amount of ascorbic acid.

The same general features occur in all experiments in air, viz. citric acid begins to accumulate

as soon as the leaves are placed in darkness; the rate of accumulation is most rapid during early stages of starvation and falls off with increasing time. In Sudan grass a time is reached when citric acid content reaches a maximum which is maintained over a considerable period, or from which there is only small decrease; in Kikuyu grass there is a relatively rapid decrease in citric acid content after attainment of the maximum value. The prolonged maximum suggests a period during which rate of formation of citric acid is equal to its rate of utilization in other reactions. Generally speaking the maximum citric acid content is reached earlier than the maximum malic acid content and during early stages of starvation citric acid increases in amount whilst malic acid content remains approximately constant or exhibits a small decrease.

TABLE 1.

Showing day on which various events occured in air.

Experiment.	End of linear protein fall.	Observed chloroplast disintegration.	Asparagine maximum.	Glutamine maximum.	Citric acid maximum.	Beginning of asparagine rise.	Beginning of malic acid rise.	Malic acid maximum.	Initial sucrose content (mg./20 gm. F.W.).
Sudan.									
I	6	6	6	6	4	1	1	6	245
IV	5	5	5	n.d.	5	1	1	const.	182
V	3	3	3	2	4	2	2	4	146
VI	7	7	7	5	5	3	3	8	409
Kikuyu.									
II	5	5	5	3	3	3	3	7	120
III	3	3	3	3	1	0	0	7	110
VII	3	3	3	n.d.	5	1	1	5	47
VIII	3	3	3	n.d.	5	1	1	5	94

It has been pointed out earlier that in our experiments sucrose content falls rapidly during early stages of starvation. The increase in amount of citric acid is most rapid during the phase of initial rapid loss of sucrose and in several experiments the citric acid maximum is coincident with the end of the rapid fall in sucrose. In Sudan grass the initial value for citric acid at the time of placing the leaves in darkness shows a linear correlation with sucrose content, but this is not the case with Kikuyu; in all cases the citric acid content is higher the greater the ontogenetic age of the leaf. It is possible therefore that quantitative changes in the systems in the leaf may occur with increasing age.

The above observations suggest a close association of citric acid formation with sugars, but do not exclude the possibility that other substances may contribute to its formation. During the period of increase in citric acid content, malic acid remains constant in amount or tends to decrease and in all cases there is an increase, sometimes small, in asparagine content. Even in Experiment VI where sucrose content was very high and little change in metabolites, with the exception of sucrose and citric acid, occurred over a period of three days there is a small increase in amount of asparagine. The possibility that malic acid or oxalacetic acid (derived from aspartic acid) may be utilized in citric acid formation must therefore be considered.

Many speculations as to the origin of citric acid in plants have been put forward (Bennet Clark, 1933) but none which consider a direct origin from a sugar can be imitated *in vitro*. On the other hand Krebs and others (Green, 1940) have shown that citric acid is produced in mammalian muscle from sugars and oxalacetic or malic acids. Krebs has put forward a scheme of oxidation of sugars wherein organic acids, including malic, oxalacetic and citric acids, act as "mediators" in hexose oxidation and are inter-related in a cycle of events each step of which is controlled by a definite enzyme system. On this view citric acid is produced by the inter-action of equivalent quantities of pyruvic and oxalacetic acids and rate of formation of citric acid will be determined by pyruvic acid content (which, as measured by sugars, falls with time in our experiments) and by oxalacetic acid (which in our experiments, as measured by asparagine or malic acid contents) increases with time. The curves of citric acid/time obtained experimentally are of the form which would be expected by the interaction of these two quantities; although they may be modified by loss of citric acid in other reactions.

It is clear in our experiments that during early stages of starvation in air rate of citric acid formation exceeds the rate of its disappearance. The fate of citric acid in plant and in moulds has been the subject of speculation (*vide* Bennet Clark, 1933) and the Krebs cycle seems to offer the most reasonable hypothesis available at present. If this cycle is operative citric acid

will be oxidized ultimately to oxalacetic acid which again combines with more pyruvic acid to reform citric acid. At each point in this cycle the rate of any individual reaction will be controlled by the value of the velocity constant and the amount of enzyme present as well as by the concentration of the organic acid substrate; it might be expected therefore that some components of the cycle (and citric acid may be such a one) are produced at a rate greater than the rate of disappearance. Another probable reaction removing citric acid must also be considered. Glutamic enzyme ("glutamic dehydrogenase"), but so far as is known not its aspartic counterpart, occurs in plants. In the presence of this enzyme and provided oxidation is going on simultaneously, α-ketoglutaric acid (the primary oxidation product of citric acid in the Krebs cycle) combines with ammonia to form glutamic acid. It has been shown in our experiments that glutamine increases in amount during the time of increase in amount of citric acid.

In conclusion, we may say that our data show that increase in amount of citric acid is associated with decrease in amount of carbohydrate and increase in amino acid and amide contents and are not inconsistent with the hypothesis that citric acid is produced from pyruvic and oxalacetic or malic acids, that it enters into oxidation through the Krebs cycle and enters, by way of its oxidation products, into amide formation.

MALIC ACID.

The picture presented by the malic acid data in the experiments described here is different from that for citric acid. Like citric acid, malic acid production is associated with presence of oxygen, for there is little significant change in malic acid content of leaves in nitrogen. In air the malic acid content is higher than the citric acid content and the maximum content of malic acid occurs later than the maximum citric acid content (*vide* Table 1).

During early stages of starvation in air whilst sucrose decreases rapidly in amount there is little change or a small decrease in malic acid content; the malic acid content then increases markedly (except in Experiment IV where the leaves were very young) and this increase is coincident with the beginning of asparagine accumulation (*vide* Table 1). Malic acid content increases to a maximum which occurs later than that of asparagine and in general thereafter decreases in amount. In Experiments II and III where l-malate was estimated by the uranyl polarimetric method no decrease occurred but we place much less reliance on this method than on the 2:4 dinitrophenylhydrazide method by which total malate was determined in all other experiments. Thoday and Jones (1939) have also indicated utilization of malate during late stages of starvation in *Kleinia*. There is no correlation of malic acid with carbohydrates among the data but an evident one exists with asparagine. Possible sources of malic acid production are from citric acid *via* the Krebs cycle or from oxalacetic acid.

Oxalacetic acid may arise from aspartic acid and it is known (Chibnall, 1939) that aspartic and glutamic acids form a large percentage of the total amino acids present in leaf proteins. Oxalacetic acid may arise from aspartic acid in at least two ways: first by the action of l-amino oxidase whereby ammonia and oxalacetic acid are produced; second by the action of the aspartic acid-aminophorase system (which is known to occur in plants) according to the equation: oxalacetic acid + amino-acid = aspartic acid + keto-acid. Malic acid may be produced from oxalacetic acid by the malic-acid-Co-enzyme II system or by interaction of α-glycerophosphate with oxalacetic acid yielding triosephosphate and malic acid. In either case equilibrium between oxalacetic acid and malic acid might be expected. Yet another source of malic acid might be through oxidation of α-ketoglutaric acid formed under the influence of glutamic acid enzyme from glutamic acid obtained by hydrolysis of protein for the equilibrium between α-ketoglutaric acid and ammonia on the one hand and glutamic acid on the other is determined by rate of oxidation of carbohydrate and as these fall, as in our experiments, α-ketoglutaric acid would increase at the expense of the glutamic acid. It has been shown earlier in this paper that the glutamine maximum is earlier than the asparagine maximum. All the above-mentioned systems are known to occur in plants and could account for the observed relations between malic acid and amides in our experiments.

It is clear from our experiments in nitrogen that little if any asparagine is derived directly from the protein molecule. During the early stages of starvation in air, amino-acids other than asparagine attain a maximum value; these amino acids contain aspartic acid. It might be expected therefore that malic acid should increase under these conditions unless it is removed in other reactions. We have already shown that citric acid is formed in greatest amount during early stages of starvation when sucrose content is relatively high and malic acid content is low and suggested that this might be due to combination of pyruvic acid with oxalacetic or malic acid. This mechanism would account for the observed constancy of malic acid content during this phase and also for the observed lag in asparagine formation, although the latter will be more dependent on rate of production of ammonia from amino acids and subsequent combination with aspartic acid. Later as sucrose decreases in amount and consequently rate of formation of citric acid becomes less, malic acid increases in amount as the asparagine content increases.

We would point out here that operation of the aspartic-acid-aminophorase system offers an explanation of the substrates used during the climacteric rise in respiration, viz. conversion of

amino acids into keto-acids with simultaneous production of asparagine; in the special case of alanine the keto-acid would be pyruvic acid, but any keto-acid might enter the oxidative cycle.

In addition to formation of malic acid from aspartic acid by aminophorases, the accumulation of malic acid after the asparagine maximum has been obtained suggests derivation from other reactions. Presumably this is formed by oxidation of aspartic acid by an amino-oxidase. Such a reaction would only become obvious on cessation of protein hydrolysis and consequent cessation of asparagine formation.

The data afford evidence that malic acid is removed by other reactions. This is clear in most experiments in late stages of starvation when malic acid decreases in amount; this stage is characterized by exhaustion of carbohydrates and cessation of amide formation, and disintegration of chloroplasts has also occurred. We have no evidence as to its fate. Linked with this loss of malic acid is the marked difference in behaviour of malic acid in young leaves (Experiment IV) compared with the older leaves of other experiments. In Experiment IV malic acid content remained approximately constant throughout the starvation period, which may be interpreted as meaning that the acid is utilized in reactions at a rate approximately equal to its rate of formation. In the absence of experimental evidence the cause of this difference remains speculative, but Marsh and Goddard (1939) have reported that in young leaves of carrot practically the whole of the enzyme system producing CO_2 is sensitive to cyanide, azide and carbon-monoxide whilst in mature leaves CO_2-production is insensitive to these reagents. It is possible therefore that quantitative and qualitative changes in enzyme systems may occur during ontogeny and may account for the observed differences.

SUGGESTED METABOLIC SCHEME IN STARVING LEAVES.

Consideration of the data presented in these experiments, and which have been discussed in the preceding sections, leads to the following suggested sequence of events:

(1) Oxidation of one or more amino acids, but including cystine, to ammonia and other compounds at a rate dependent on the sucrose content.

(2) Hydrolysis of protein to restore equilibrium among amino acids, especially of sulphur-rich proteins and including proteins of the chloroplast. Hydrolysis of chloroplast protein is accompanied by decrease in amounts of chlorophyll and of ascorbic acid.

The following four processes occur simultaneously and are associated with the climacteric rise in respiration:

(3) Formation of glutamine (a) from ammonia (derived from oxidation of amino acids) and glutamic acid (derived from the protein molecule) at a rate depending on rate of oxidation of amino acids and (b) from α-ketoglutaric acid and ammonia in the presence of glutamic enzyme, the equilibrium position being determined by rate of oxidation of carbohydrates in the system.

(4) Formation of asparagine from ammonia and aspartic acid derived from protein hydrolysis.

(5) Production of citric acid from pyruvic acid (derived from glycolysis of sugars) and oxalacetic or malic acids at a rate determined by the sucrose and oxalacetic acid contents; and its utilization by oxidation through the Krebs cycle or in formation of glutamic acid.

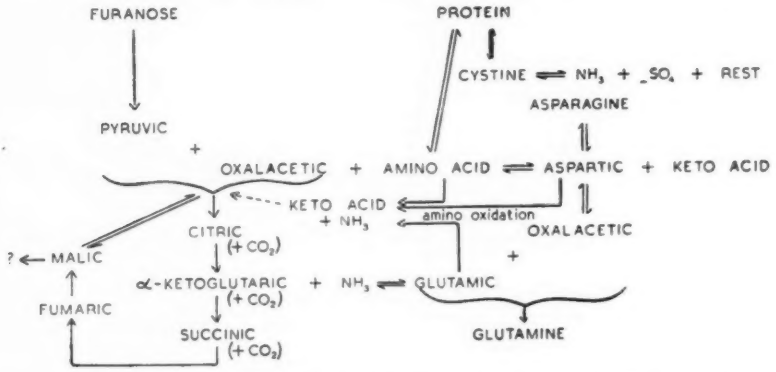
(6) Production of oxalacetic acid from aspartic acid by aspartic acid aminophorase, by oxidation of citric acid or by oxidation of α-ketoglutaric acid derived from glutamic acid; and production of malic acid in equilibrium with this.

(7) As as result of falling respiration rate α-ketoglutaric acid increases at the expense of glutamic acid. Malic and oxalacetic acids increase by oxidation of α-ketoglutaric acid and further production of asparagine from oxalacetic acid at expense of amino acids by action of aminophorase. Possible transamination of glutamic acid and oxalacetic acid to aspartic acid may also occur under the influence of glutamic aminophorase.

(8) Cessation of protein synthesis due to exhaustion of sulphur-rich proteins and breakdown of chloroplast structure resulting in cessation of amino acid production and of accumulation of amides.

(9) Oxidation of amino acids and amides, probably by l-amino oxidases and which possibly has been continuous, becomes obvious and ammonia accumulates. Disappearance of citric and malic acids in undetermined ways.

The suggested sequence of reactions is summarized in the following scheme.



Schemes analogous to that proposed here have been suggested by Wood (1941, 1942) to account for the reactions which occur during the life history of a grass plant and for those constituting the synthetic phase of protein metabolism.

SUMMARY.

Details are given of eight experiments in which leaves of Sudan Grass (*Andropogen sudanensis*, L. and B.) and Kikuyu Grass (*Pennisetum clandestinum*, Hockst.) were placed under starvation conditions both in air and in nitrogen. Observations which are essentially new include the following:

During starvation in air chloroplast protein is broken down into soluble products. In Sudan Grass chloroplast-protein breaks down more rapidly than cytoplasmic protein, but in Kikuyu both chloroplast and cytoplasmic proteins break down at approximately equal rates. Decrease in amount of protein with time is approximately linear until a point is reached, coincident with cessation of yellowing, when there is little further decrease in protein.

Decrease in amounts of chlorophyll, ascorbic acid and dehydroascorbic acid is associated with decrease in chloroplast protein.

Cessation of protein hydrolysis in the leaves is associated with disintegration of the chloroplast structure.

In air onset of protein breakdown is determined by the initial sucrose content; and residual amino acids, glutamine, asparagine and ammonia successively attain maximum contents, but their formation commences with commencement of protein breakdown. The maximum asparagine content coincides with cessation of protein loss.

In nitrogen amount of protein degradation is very small compared with that in air and amino acids only, but not amides and ammonia, accumulate in the leaves. On transferring the leaves to air proteins decrease in amount and production of amides and ammonia takes place.

In air respiration rate initially decreases with falling sucrose content, but this is followed by a climacteric rise which occurs between the times of maximum

residual amino-acid content and maximum asparagine content. The onset of the climacteric appears to be determined by the initial carbohydrate content and its form by the sucrose and amino acid contents.

In nitrogen no climacteric rise in respiration rate occurs. The sucrose content decreases more rapidly than in air and the form of the respiration rate/time curve is similar to that for sucrose content/time.

Malic and citric acid contents exhibit little change in nitrogen.

In air citric acid increases during early stages of starvation but later reaches a steady value or decreases in amount. Increase in citric acid is associated with decrease in sucrose content and increase in amino and asparagine content.

In air malic acid content at first remains approximately constant but later increases markedly in amount. Increase in malic acid content is associated with marked increase in asparagine content. Later still, malic acid decreases in amount.

Inter-relations between the various metabolites are discussed and a scheme showing the possible course of metabolism during starvation is proposed.

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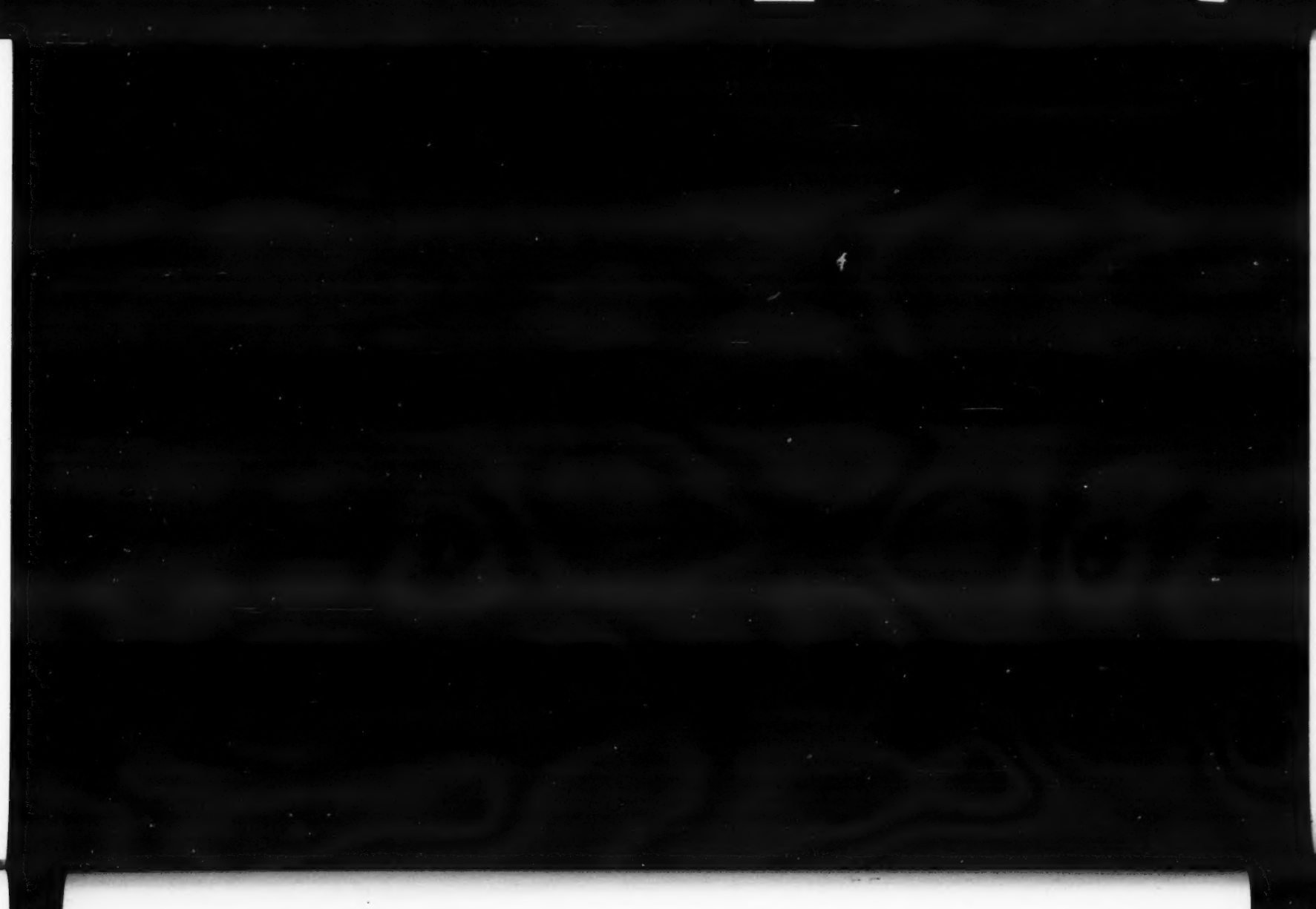
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